Distribution and function of the oestrogen receptor ß in the cardiovascular system of the mouse

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Declaration

I hereby declare that the work described here in this thesis was performed entirely by myself, except for procedures acknowledged in the text. This work contains no material that has been accepted for the award of any other degree or diploma in any university or tertiary institution and to the best of my knowledge contains no material previously published or written by any other person, except where stated in the text.

Katrina Frances Ness

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Abstract

Oestrogen is suggested to have a protective role within the cardiovascular system as the risk of coronary heart disease in pre- menopausal women is lower than that of age-matched males and this protection is lost following the menopause. Oestrogen is known to act via two receptor subtypes, ERα and ERß. However, little is known as to the role of either receptor in mediating the effects of oestrogen in the cardiovascular system (CVS).

The aim of this thesis was to determine the distribution of ERs in the CVS and whether this was altered under pathophysiological conditions. We hypothesised that oestrogen acting through ERs modifies vascular function and contributes to regulation of blood pressure. Therefore, blood pressure and vascular function were assessed in female mice of different oestrogen status and the influence of ERß determined by studying male ERß knock-out (ßERKO) mice and female WT mice treated with a novel ERß antagonist.

Immunoreactive ERß was associated with nuclei of myocardial, endothelial (ETC) and vascular smooth muscle cells (VSMC) of the heart, aorta and mesenteric artery in both male and female wild type (WT) and ßERKO mice. Immunoreactive ERß was also expressed in the ßERKO mouse, which suggests the presence of part of the receptor. However it was shown not to function as native WT ERß protein as the ovarian phenotype of the ßERKO was consistent with loss of function. The distribution of ERß in mouse models of atherosclerosis and myocardial infarction (MI) were not different to that of normal mice.

Mean arterial blood pressure (MABP) measured in young and aged WT and ßERKO mice, by implantation of a Millar catheter into the left carotid artery, was found to increase with age. However, there was no difference in MABP between ßERKO and age- matched WT animals. The aorta isolated from these animals demonstrated that the loss of functional ERß led to increased sensitivity to the α_1- adrenoceptor agonist, phenylephrine (PE) relative to their age- matched WT controls. Incubation with an

inhibitor of nitric oxide (NO) synthase suggests that this may be due to a reduction in the basal release of NO in the ßERKO. The endothelium- dependent and -independent relaxation responses were not different between ßERKO mice and age-matched WT controls.

MABP of female WT mice, as measured by radiotelemetry suggested that both endogenous and exogenous oestrogen increased MABP. Treatment of female mice with an ERß antagonist resulted in an increase in MABP in sham ovx mice whilst MABP was reduced in mice which were of ovx+E. Mesenteric vessels isolated from sham ovx and ovx+E showed that chronic E treatment increased the sensitivity to PE. Treatment of sham ovx animals with an ER antagonist did not alter vascular function. Whilst treatment of ovx+E mice with the ERß antagonist, led to a reduction in the sensitivity to PE and an increase in both endothelium- dependent and -independent vasodilatation.

In conclusion, ERß is widely expressed in the CVS, of both male and female mice and was not altered in animal models of MI or atherosclerosis. In the vasculature of both male and female mice ERß modulated vascular function. The loss of ERß function, in female mice with endogenous oestrogen, led to an increase in MABP which can be confirmed for the first time using a novel ERß antagonist.

Publications and Presentations

Abstracts and presentations

Ness K.F., Sharif I, Macpherson S, Gustaffson J-A, Saunders P.T.K., Gray G.A. (2002) 'Modification of vascular function by the oestrogen receptor β: the effect of ageing.' Oral presentation at the British Pharmacological Society, Glasgow, September 2002.

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List of Abbreviations

A

ACh	- Acetylcholine
ANOVA	- Analysis of Variance
Apo E-/-	- Apolipoprotein E knock- out mouse

B

BP	- Blood Pressure
bp	- base pair
BSA	- bovine serum albumin
ßERKO	- oestrogen receptor beta knock out mouse

C

$CaCl_2$	- Calcium chloride
CAL	- Coronary artery ligation
cDNA	- complimentary deoxyribonucleic acid
COX	- cyclooxygenase
CRC	- concentration response curve

D

DAB	- 3-3'-diaminobenzidine
dH_2O	- distilled deionised water
DNA	- deoxyribo-nucleic acid
dNTP	- deoxynucleotide triphosphate

E

E	- oestrogen

EDTA	- ethylene diamine tetra-acetate
EF	- ejection fraction
ER	- oestrogen receptor
ERKO	- oestrogen receptor alpha knock out mouse
ERα	- oestrogen receptor alpha
ERß	- oestrogen receptor beta

F

FS	- Fractional shortening

G

g	- centrifugal force
G	- gauge

H

H_2O_2	- hydrogen peroxide
hr(s)	- hour(s)
5-HT	- 5-hydroxytryptamine

I

i.p.	- intra-peritoneal

K

KCl	- potassium chloride

L

LVEDD	- left ventricular end diastolic diameter
LVESD	- left ventricular end systolic diameter

L-NAME - N-nitro-L-arginine methyl ester

M

min(s) - minute(s)

MI - myocardial infarction

mN - milli- newtons

mRNA - messenger RNA

N

NA - noradrenaline

O

ovx - ovariectomy

ovx - E - ovariectomy with placebo supplementation

ovx + E - ovariectomy with oestrogen supplementation

P

PCR - polymerase chain reaction

PE - phenylephrine

R

RNA - ribonucleic acid

RT-PCR - reverse transcription polymerase chain reaction

S

s.c. - subcutaneous

SDS - sodium dodecyl sulphate

SNP - sodium nitroprusside

T

TBS	- tris- buffered saline
TE	- Tris- EDTA

U

UV	- ultraviolet (light)

V

V	- volts

W

WT	- wild type

Chapter 1

Introduction

1.1 Oestrogen synthesis

In pre-menopausal females the principal site for oestrogen biosynthesis are the ovaries. The ovaries have the ability to synthesise C19 precursors and to produce and secrete oestradiol, *Figure 1.1*, following stimulation by the gonadotrophins, follicle stimulating hormone (FSH) and lutenizing hormone (LH). At specific points in the oestrous cycle, FSH and LH are released from the anterior pituitary in response to gonadotrophin releasing hormone (GnRH) which is released from the hypothalamus, *Figure 1.2*. FSH acts directly on granulosa cells to stimulate follicular growth by synthesising androgens which are converted to oestrogen, this is known as the follicular phase which terminates at ovulation. LH contributes to follicular growth by acting on theca cells within the ovary to synthesis androgens which are then converted to oestrogen via aromatase in granulosa cells. The surge in LH concentrations, due to the positive feedback of moderate levels of oestrogen, results in ovulation and the transformation of remaining granulosa and theca cells of that follicle into the corpus luteum which secretes oestrogen and progesterone which act on the hypothalamus to suppress the release of GnRH and subsequently FSH and LH, this is known as the luteal phase.

However, in post- menopausal women, when the ovaries have ceased to synthesis oestrogen and also in men, significant amounts of oestrogens are produced in a number of extragonadal sites. Such sites of oestrogen biosynthesis include mesenchymal cells of adipose tissue, osteoblasts of bone and vascular endothelial as well as aortic smooth muscle cells (reviewed in Simpson *et al*, 2000). However, in these cells the process of oestrogen biosynthesis differs to that of the ovary as these tissues are dependent on circulating C19 androgenic precursors as they are unable to convert cholesterol to C19 precursors. Although the amounts of oestrogens synthesised at these sites may be low the local tissue concentrations are likely to be high and produce physiological effects either by acting in a paracrine or autocrine manner. The importance of extragonadal synthesis of oestrogen and its role in males was highlighted by the finding that men with a mutation in the gene coding for

aromatase had reduced bone mass and their bones were incapable of epiphyseal fusion (Carani *et al*, 1997).

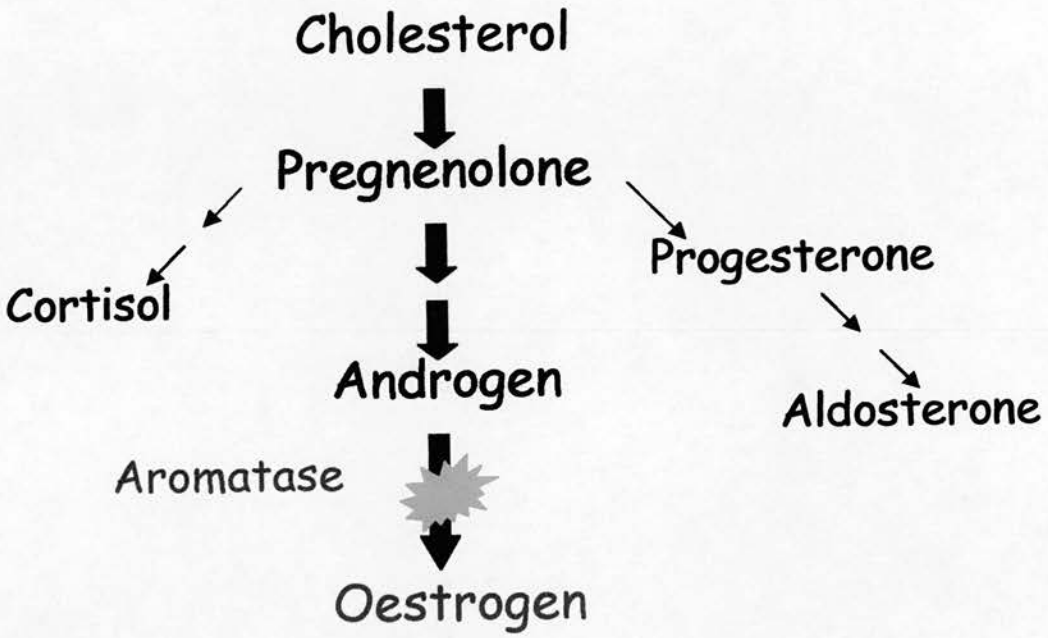

Figure 1.1 Biosynthesis of oestrogen from the steroid precursor cholesterol

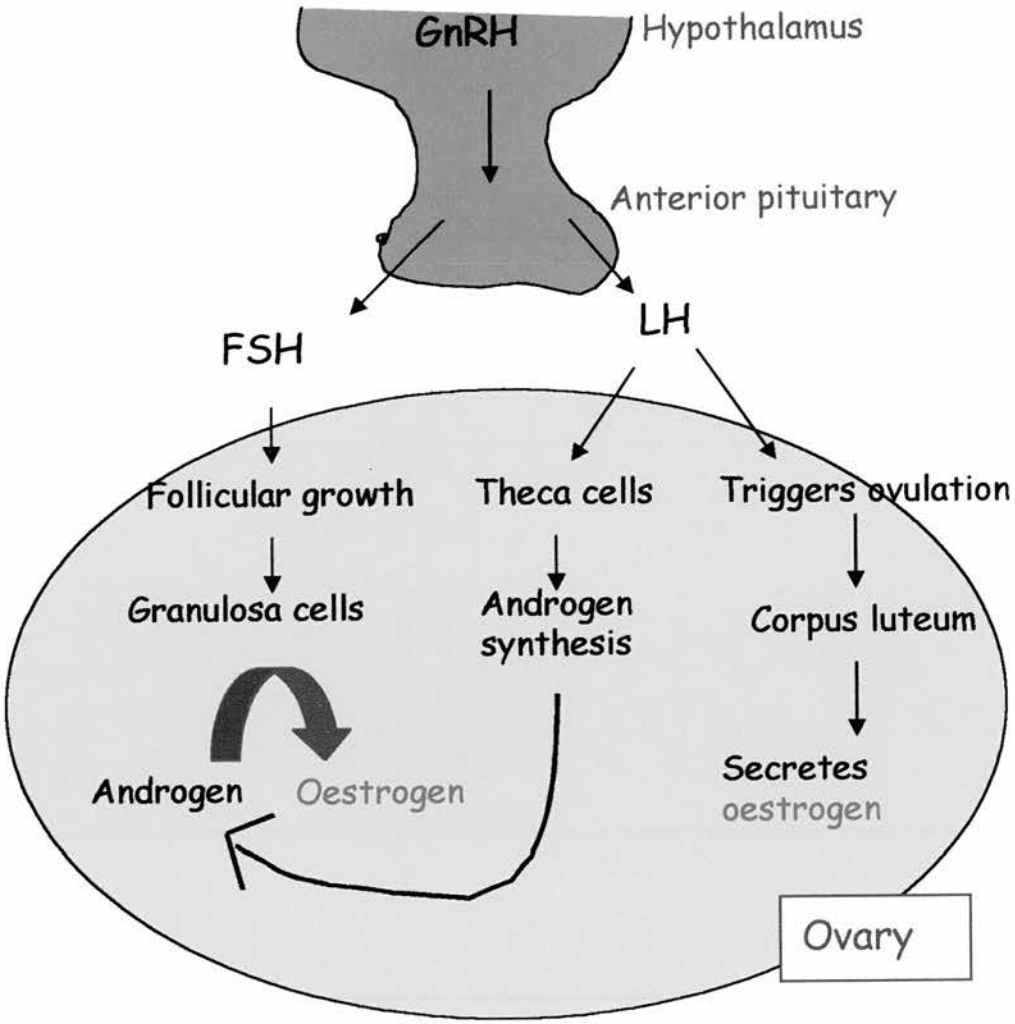

Figure 1.2 Activation of oestrogen biosynthesis in the ovary by hypothalamic and pituitary hormones.

1.2 Structure of oestrogen receptors and mechanisms of oestrogen receptor signalling

1.2.1 Oestrogen receptor structure

Oestrogen exerts its effect by binding to classical steroid hormone receptors, the oestrogen receptor (ER) α (Green *et al*, 1986) and the more recently described ERß (Kuiper *et al*, 1996). Similar to other receptors within the steroid hormone receptor superfamily, ERα and ß, which share a high degree of homology, consist of three functional domains; the N- terminal (A/ B domains), the DNA binding domain (C domain) and the ligand binding domain or C-terminal (E/ F domain) (reviewed in Nilsson *et al*, 2001, Kuiper *et al*, 1996; Kuiper and Gustafsson, 1997b), *Figure 1.3*.

The N-terminal domain, in which there is the greatest degree of sequence diversity between ERα and ERß, contains the ligand-independent activation function (AF-1) site which is a target site for receptor phosphorylation, is involved in protein-protein interactions and is the site at which other transcription factors interact with the receptor (reviewed in Nilsson *et al*, 2001). The N-terminus of ERß is well conserved between species implying that there is a degree of evolutionary constraint and suggests that this receptor is of functional importance. The structural differences between these receptors may explain the differences observed between the transcriptional activation properties of these receptors. Delaunay *et al*, demonstrated, by fusing the amino terminus of the ERß protein to a heterologous yeast DNA binding domain, that synergism between the amino and carboxyl terminus of ERß was required for transcriptional activation (2000). This was in contrast to ERα, of which the amino terminus was sufficient for transcription in certain cell types (Metzger *et al*, 1995). Furthermore, although 17ß-oestradiol binds to both receptors with similar affinity (Kuiper *et al*, 1997) the transcriptional response of ERα in some cell types was greater than that of ERß (McInerney *et al*, 1996). 4-hydroxytamoxifen which was shown to be an ERß antagonist, is an agonist for ERα on a promoter on which transcription can be regulated by ERα AF-1 alone (Berry *et al*, 1990).

Therefore, this suggests that due to the structural differences between the amino terminals, ERβ has weaker transcriptional activity than ERα.

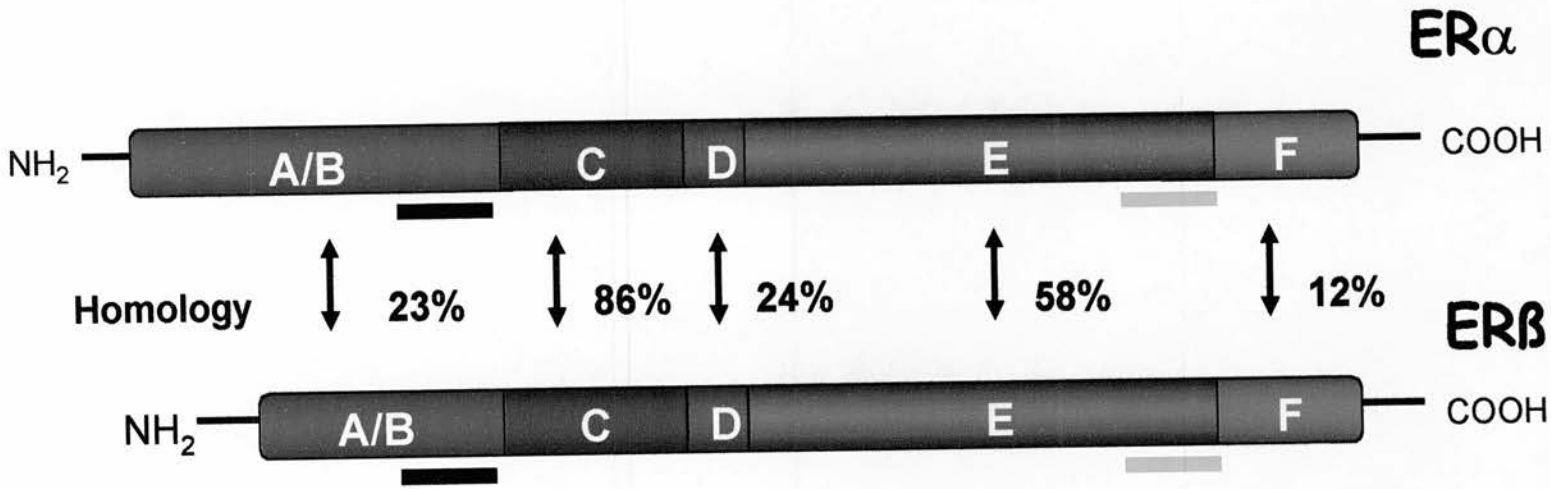

Figure 1.3 ***Schematic diagram of the regions and homology between ERs.***

The figure indicates the distinct domains of both ERα and ERß and the numbers indicate the degree of homology shared between the domains of both ERs. The domains of the receptors are labelled from the N-terminus; A/B; which contains the AF1 site —, *C; DNA binding domain, D; hinge region, E and F; the ligand binding domains which contain the AF-2 site* .

The DNA binding domain (DBD) is the region in which ERα and ERβ share the highest degree of homology (86%). The DBD is important for receptor dimerisation and binding to specific pallindromic DNA sequences, oestrogen response elements (ERE) located within target genes (reviewed in Nilsson *et al*, 2001). Therefore ERα and ERß will interact with ERE of various genes with similar affinity and specificity.

The ligand binding domain (LBD) is the region of the oestrogen receptor which binds hormone or ligand. This region of the receptor also mediates receptor dimerisation (either forming homodimers or heterodimers between receptors), nuclear translocation of the receptor (reviewed in Nilsson *et al*, 2001 and Hall *et al,* 2001) and recruitment of and interaction with coregulators via AF-2. ERα and ERβ share 58% homology in the primary amino acid sequence in the LBD with high homology in the ligand binding cavity of both ERs (Brzozowski *et al*, 1997). However, some ligands have been reported to bind with a higher affinity to ERα than ERβ and vice versa (Kuiper *et al*, 1997). It was suggested that this was due to differences in the tertiary structure of the ligand binding cavity of ERα and ERβ despite sharing homology in their primary amino acid sequences. The gene modulatory effect of the ER after ligand binding depends in part on the conformational change in the LBD of the receptor leading to the formation of a hydrophobic groove, known as AF-2, which enables cofactors to bind (reviewed Ruff *et al*, 2000). The conformational change in the ER following ligand binding promotes and stabilises ER cofactor interaction which subsequently stabilises ER ligand interaction which thereby slows the rate of dissociation. Cofactors can either be co-activators or co-repressors (reviewed Nilsson *et al*, 2001). Co-activators are implicated in transcriptional activation of the target gene and can bridge the ER to either histones, or components of basal transcription or both. Alternatively, co-repressors either associate with the ER in the presence of antagonist or attenuate transcription of target genes by competing with co-activators for binding with AF-2. Similar to the differences that exist between ERα and ERβ in the affinity for different ligands (Kuiper *et al*, 1997) there also exist differences in the binding affinity of the AF-2 domain for specific cofactors between both receptors (reviewed Nilsson *et al*, 2001). One such example is that ERα is less efficient than ERβ in

recruiting the co-activator complex TRAP/DRIP, which connects directly to basal transcription machinery. This suggests that the ligand binding cavity and the AF-2 domain may provide a site for the targeting of ER specific agonists/ antagonists.

1.2.2 Oestrogen receptor signalling

For many years it was believed that oestrogen only signalled through the classical ligand-dependent and genomic pathway. Recently however, several other signalling pathways have been suggested. These include an ERE-independent mechanism where the activated ER can alter transcription of other response elements by binding DNA-bound transcription factors at AF-1 binding sites. Alternatively, there may be non-genomic signalling where the ER homo or heterodimers is present in, or close to, the cell plasma membrane, which upon ligand binding leads to the activation of intracellular signalling cascades (reviewed in Kelly and Levin, 2001 Mendelsohn 2002).

1.2.2.1 Classical ER signalling pathway.

In the classical signalling pathway, it was believed that ERs were rapidly transferred to the nucleus only upon ligand binding. However, it is now confirmed that ER are predominantly nuclear proteins and are located in the cell nucleus whether or not it is bound with a ligand (Parker, 1995). The ER is stored in the nucleus in the inactive state (King and Greene, 1984), bound to a heat shock protein which dissociates on binding of the ligand and activation of the receptor. The binding of the agonist leads to homo- or hetero- dimerisation of ERs and induces a conformational change such that the DBD of the ER dimers bind to ERE in the promoter region of target genes, (reviewed in Hall *et al*, 2001), *Figure 1.4*. As described in *section 1.2.1,* the conformational change upon ligand binding allows and stabilises the binding of coactivators to AF-2 within the carboxy terminal, which subsequently stabilises ER- ligand binding (Gee *et al*, 1999) such that it may result in maximal transcriptional activity. The importance of coactivators in steroid

hormone function was highlighted in mice which were lacking steroid receptor coactivator-1, which demonstrated decreased response to hormones in the prostate and uterus (Xu *et al*, 1998). Opposite to the function of coactivators are corepressors, such as repressor for oestrogen activity (REA), which function to silence the activity of ERs (Montano *et al*, 1999). Therefore, coactivators and corepressors expressed in a given cell type may determine the extent of transcriptional activity of the ligand activated ER.

1.2.2.2 Nongenomic actions of oestrogen

The observation that oestrogen treatments could produce effects that were too rapid for the activation of RNA and protein synthesis suggested that oestrogen may have nongenomic effects, *Figure 1.4*.

The non-genomic effects of oestrogen have been disputed as ER do not express sequences that could code for a hydrophobic, transmembrane spanning region. However, it is possible that ERs undergo post-translational modification such that they can be transported and localised in the cell membrane. It was shown in Chinese hamster ovary cells that were transfected with ERα and ERß that both of these receptors were located in the nucleus and cell membrane (Razandi *et al*, 2000). Although ERs lack myristoylation and palmitoylation sites, which could anchor them to the plasma membrane, they may be tethered to the membrane by glycophosphatidly- inositol (GPI) links or be localised to caveolae, which are signalling transduction and vesicular trafficking domains in the plasma membrane (reviewed in Kelly and Levin, 2001). Oestrogen rapidly activated eNOS in cultured endothelial cells in which ERα reportedly coprecipitated with endothelial cell caveolae (Shaul *et al*, 1996, Shaul *et al,* 1998). This was shown to be ER mediated as the rapid activation of eNOS was completely inhibited by the ER antagonist ICI 182 780. The exact signalling pathway by which ERα activates eNOS has yet to be fully determined. However, ERα has been shown to activate eNOS in a non-genomic manner via interaction with the phosphatidylinositol 3-OH kinase pathway and the MAPkinase pathway in endothelial cells (reviewed in Simoncini *et al*, 2002 and Ho and

Liao, 2002). Furthermore, a recent study by Song *et al*, demonstrated that ERα interacts with the adaptor protein Shc such that it forms a complex with IGF-1R. This complex can then be translocated to the cell membrane and induce the phosphorylation of MAPkinase and subsequent downstream signalling. (Song *et al*, 2003).

Although evidence suggests that oestrogen may produce rapid effects via ERα, whether ERß can also mediate rapid non-genomic effects requires further exploration.

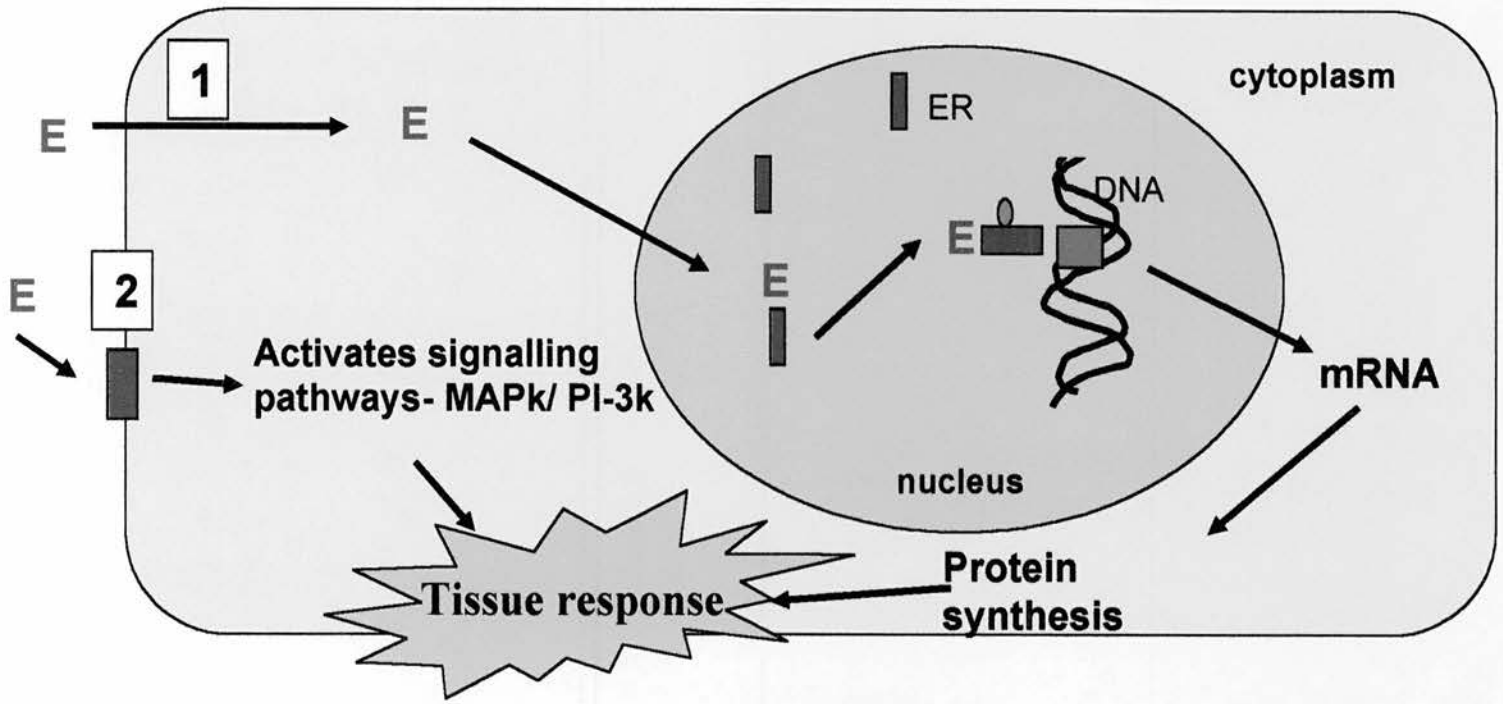

Figure 1.4 ***Model of the genomic and nongenomic mechanisms of ER signalling***

1) Classical genomic signalling pathway; oestrogen (E) diffuses across the cell membrane and enters the nucleus where it binds to ERs. The ER undergoes a conformational change enabling it to bind to the oestrogen response element (ERE) and interact with coactivators which enhance transcription initiated by the binding of the activated ER to the ERE. 2) Nongenomic signalling; oestrogen interacts with ER located on the cell membrane and activates a series of intracellular signalling pathways generating a rapid tissue response. ▭ *; ERE,* ◯*; coactivator*

1.3 Oestrogen receptor function

By 1996 it was clear that two receptor molecules for oestrogen existed, both of which shared a high degree of homology, specifically within regions of the LBD (reviewed in Nilsson *et al*, 2001). Studies to date aimed at elucidating the role(s) played by ERα and ERß induced changes in gene expression have been hampered by the lack of subtype specific receptor antagonists.

However, this was overcome firstly, by gene deletion techniques by which ER subtype specific knock out mice were generated; ERα knock-out (ERKO, Lubhan *et al*, 1993) and ERß knock-out, (ßERKO, Krege *et al*, 1998). The generation of these mice provided a useful tool in studying the effects of oestrogen mediated via these individual receptors. Secondly, by the recent development of ER subtype specific agonists and antagonists as a result of improved understanding of the structure and function of the individual regions of the ERs. Examples of such drugs are ERα agonist, propyl pyrazole (PPT, Stauffer *et al*, 2000) and diarylpropionitrile (DPN, Meyers *et al*, 2001), a selective ERß agonist. These drugs have a 410 fold and 70 fold higher binding affinity for ERα and ERß respectively.

1.3.1 ER knock out mice

Studies on the reproductive systems of ER knock-out mice have demonstrated that both of these receptors are of physiological importance in maintaining normal fertility. Both sexes of the ERKO mouse were shown to be infertile (Lubhan *et al*, 1993). The ovary of the ERKO females was enlarged, hemorrhagic and consisted of cystic follicles (Schomberg *et al*, 1999). The ovaries from these mice also lacked corpus luteum which suggests that the ovaries are incapable of spontaneous ovulation. Additional studies suggested that the ovarian phenotype of the ERKO mouse, was exacerbated by the elevated levels of gonadotrophins that existed in these animals (Sharkey *et al*, 1999). The testes of the male ERKO were reduced in size and male infertility was attributed to a defect in their ability to resorb fluid within the efferent ductules which affected sperm motility such that these sperm

were unable to fertilise wild-type oocytes *in- vitro* (reviewed in Couse and Korach, 1999). In contrast, female ßERKO mice are capable of producing litters although the number of litters and the number of pups were substantially fewer than that of wild-type animals. The subfertility of ßERKO females may be due to an increase in early atretic follicles and a reduction in the number of corpus luteum (Krege *et al*, 1998). In contrast to the ERKO, ßERKO males were found to be fully fertile.

Mice in which the ER genes have been modified have been used to study the role of these receptors in mediating the effects of oestrogen in the cardiovascular system and this will be discussed later in this chapter.

1.3.2 ER selective agonists

ER selective agonists have been described in the literature (Harrington *et al*, 2003) but have only recently become commercially available. To date only limited research has been published using ER selective agonists as tools to study the roles of the different ER subtypes in mediating the effects of oestrogens. The advantage in using selective agonists over 'knock-out animals' is that 'knock-out' animals have passed through all stages of development without the presence of the respective receptor. Therefore, the 'knock out' mouse cannot be considered different to the wild-type mouse only in the lack of expressing the receptor, as some compensatory mechanisms during development cannot be excluded. Furthermore, the method of gene deletion employed to generate the ERKO and ßERKO animals can result in aberrant splicing of the disrupted gene and therefore the 'knock-out' generated may not be a complete null. Literature has reported that such variants were detected in both ERKO and ßERKO mice (Couse *et al*, 1995, Krege *et al*, 1998). However, the variants expressed in these models were not found to be the same as transcribed wild-type mRNA

Early studies using the ER selective agonists have demonstrated that activation of ERß in the uterus by DPN downregulates the expression of the progesterone receptor (PR, Frasor *et al*, 2003). These findings are consistent with results of a study using

ßERKO mice in which expression of PR mRNA in the uterus was upregulated following chronic oestrogen treatment (Weihua *et al*, 2000), this suggests that ERß attenuates the expression of PR. The selective ERα agonist, PPT has also been shown to have effects *in vivo*; treatment caused an increase in PR expression in the cerebellum showing PPT is capable of crossing the blood brain barrier (Harris *et al*, 2002). Other studies using DPN have demonstrated that neuroprotection provided by oestradiol following global ischaemia is likely to be mediated by ERß (Carswell *et al*, 2004).

Few studies have been published in the literature reporting the effects of ER selective agonists in the cardiovascular system. This is an area of research that needs to expand over the next few years in order to improve the safety and efficiency of oestrogens in cardiovascular disease. Studies published to date have demonstrated that in spontaneously hypertensive rats (SHR) treatment with an ERα agonist prevented endothelial dysfunction, and that stimulation of ERα increased eNOS protein expression and vasodilator-stimulated phosphoprotein phosphorylation (Widder *et al*, 2003).

1.3.3 ER in the cardiovascular system

In 1996 when ERß was identified and immediately thereafter, research rapidly opened up in the areas of both genomic and non-genomic effects of oestrogen and the role of oestrogen receptors in mediating those effects in the cardiovascular system. In addition, a number of studies were undertaken to determine the distribution of both receptors in the cardiovascular system of both humans and animals models.

1.3.3.1 ER distribution.

Oestrogen action is dependent upon the presence of specific ligand-activated receptors in target tissues. Therefore prior to determining the effects of oestrogen on

target cells, studies in receptor distribution can provide data as to whether cells are likely to provide a future therapeutic target.

Following the identification of ERα and ERß (Green *et al*, 1986, Kuiper *et al*, 1996), immunohistochemical and molecular biological techniques were employed to determine their distribution in the cardiovascular system (Orimo *et al,* 1993, Leiberman *et al*, 1990, Karas *et al*, 1994, Iafrati *et al*, 1997). ERα was reported to be expressed in rat aortic smooth muscle cells (Orimo *et al,* 1993) and also human vascular smooth muscle cells (Bausero *et al*, 2000). However, it became apparent that expression of ERα varied with both the species and vascular bed studied. Although expressed in both human and rat vascular smooth muscle cells, it was reported that VSMC of both the carotid artery and aorta of the guinea-pig lacked immunoreactive ERα (Leiberman *et al*, 1990). It is possible that the variation in expression of ERα in the vasculature may account for some of the differences reported between species in the functional effects of oestrogen. For example, there have been reports of differences in vascular responses between rabbit and rat (Paredes-Carbajal *et al*, 1995). Therefore determining the expression of ERs in the animal model and vascular model being studied may be crucial so that appropriate comparisons may be made between reports in the literature. Literature published on the distribution of ERα in the cardiovascular system has mainly involved the use of whole tissue extracts combined with Western analysis or detection of mRNA. Determination of mRNA expression using whole blood vessels means that whether ERα is specifically expressed in vascular smooth muscle or endothelial cells is not known. Where immunocytochemistry has been applied, it was to cultured cells, either smooth muscle or endothelial cells. Although cellular localisation of the protein to the nucleus was demonstrated compatible studies on intact tissue were not undertaken. Studies to assess the presence of ERα in coronary arteries of monkeys and humans have shown that nuclei of both endothelial cells and vascular smooth muscle cells express ERα (Diano *et al*, 1999). However, the vascular bed studied may result in differing results as studies in the vascular endothelium of human and non-human primate endometrium have shown that these cells express ERß but not ERα (Critchley *et al*, 2001).

Immunocytochemical analysis of female rat vascular beds localised the expression of ERß protein in the nuclei of vascular smooth muscle and endothelial cells of the aorta, tail and uterine arteries (Andersson *et al*, 2001). Several studies have reported the expression of mRNA for ERß in the blood vessels of rat and mice (Lindner *et al*, 1998, Makela *et al*, 1999). However, despite the use of mice, in particular ERKO and ßERKO mice as tools to extrapolate the influence of oestrogen and the mechanisms by which these effects are produced, it is only very recently that literature has reported the expression of ERs in the mouse vasculature. Initial studies by Lang *et al*, reported that ERß but not ERα was expressed in the mouse aorta (Liang *et al*, 2001).

Both ERα and ERß are known to be expressed in the nuclei of cardiomyocytes and coronary arteries of both rat and rabbit hearts (Lou *et al*, 1998, Saunders *et al*, 1997, Neudling *et al*, 2001) as studied using whole tissue and cultured rat cardiomyocytes. However, the expression of ERs, either RNA or protein, in cardiomyocytes has not yet been reported in the mouse.

1.4 Influence of oestrogen in the cardiovascular system.

1.4.1 Early observations and clinical trials

The role of oestrogen is not restricted to the reproductive system but also extends to masculinisation of the brain, closure of epiphyseal plates and the function of the cardiovascular system (reviewed in Nilsson *et al*, 2001).

Oestrogen was suggested to have a protective role in the cardiovascular system as women suffer less from cardiovascular disease during their reproductive life than age-matched men (Benetos *et al,* 1999). In addition to the described benefits of oestrogen on coronary heart disease and stroke, one other beneficial effect of oestrogen was believed to be on the regulation of blood pressure. It was suggested that oestrogen lowered blood pressure as premenopausal women had a lower systolic and diastolic blood pressure than those women that had either undergone natural or

surgically induced menopause (Stassen *et al*, 1989, Seely *et al*, 1999, Cagnacci *et al*, 1999). Furthermore, this apparent protection gradually declines after the menopause as circulating oestrogen levels fall.

Oestrogen therapy was used as a treatment for menopausal symptoms from the late 1940s and since then its effects out with the reproductive system have been observed. Observational studies reported that women receiving oestrogen replacement therapy had a 50% reduction in the incidence of secondary heart attacks (Sullivan *et al*, 1990). It is believed that oestrogen mediates its cardioprotective effects by providing a favourable lipid profile (Kushawaha, 1992) and by enhancing the release and activity of endothelium derived nitric oxide (EDNO) (Kauser and Rubanyi, 1997, Hayashi *et al*, 1994). Despite the beneficial effects of oestrogen reported from observational studies, recently, randomised clinical trials (Hulley *et al*, 1998) have shown that hormone replacement therapy (HRT) had no overall benefit in coronary heart disease. The HERS trial (Hulley *et al*, 1998) was stopped prematurely as during the first year on HRT, there was a small but significant increase in the risk of acute myocardial infarction. This increased risk occurred mostly, but not uniquely, in women who already had established coronary heart disease. However, the early termination of the trial was surprising as a trend was developing that cardiovascular health was improving following long term treatment, as the risk was found to decrease following 4-5 years treatment in the hormone replacement group. In 2002 the Women's Health Intiative (WHI) was published, this was a randomised controlled study of asymptomatic healthy post-menopausal women, reporting that combined oestrogen treatment was associated with a slight increase in the absolute risk of stroke, heart attacks and breast cancer. However, this trial has been recently met with criticism of a possible detection bias such as the unblinding of the study due to the onset of symptoms associated with hormone replacement. Additionally, following the publication of the results of the small increase in acute MI detected in the HERS trial letters were sent to all participants of the WHI trial informing them of the increase risk during the first study year which may have led to otherwise clinically unrecognised MIs being diagnosed more often (Garbe and Suissa *et al*, 2004).

1.4.2 Effects of oestrogen on the myocardium

Although the HERS trial, which indicated that oestrogen supplementation did not provide any overall benefit in CHD, a subsequent study published by Hodis *et al*, showed that healthy postmenopausal women treated with unopposed oestrogen, ie not combined with progesterone, had a slower rate of coronary arteriosclerosis than those treated with placebo (Hodis *et al*, 2001). The ESPRIT study which followed shortly after the study by Hodis *et al*, was designed to assess whether unopposed oestrogen provided protection against a second cardiac event or death in postmenopausal women who had just suffered a first ischaemic event (Cherry *et al*, 2002). Although, a reduction in cardiac death was observed in women receiving oestrogen supplementation it did not reach significance as the total death rate from all causes of death was also reduced in this group. Therefore, despite the observation that oestrogen had some beneficial effects on the rate of coronary arteriosclerosis, a beneficial effect was not observed in those women who had previously suffered a coronary event.

The myocardium relies on nutrients and oxygen being delivered by coronary arteries, if these arteries become occluded, which may occur by the development of an atherosclerotic plaque, blood flow would be obstructed. Therefore, the demands of the myocardium are no longer being met and tissue may become ischaemic as a result of hypoxia. If the ischaemic area of the myocardium is not reperfused it will result in apoptosis and myocardial infarction (MI). However, the process of restoring blood flow to the ischaemic area, can in itself result in tissue damage. The reintroduction of oxygen during reperfusion to the ischaemic site can result in the development of free radicals that can led to cellular damage and death. In a canine model of ischemia reperfusion, chronic treatment with oestradiol attenuated reperfusion arrhythmias, myocardial systolic dysfunction and the temporary impairment of endothelial function (Kim *et al*, 1996). Although oestrogen has beneficial effects on lipoprotein metabolism these account for only about 25- 40% of its beneficial effects (Barrett-Connor and Bush, 1991) and in the study by Kim *et al*, it was suggested that the protection provided by oestrogen was in part due to its

antioxidant effects. Further studies also demonstrated a role for the anti-inflammatory effects of oestrogen in reducing leukocyte accumulation as a result of myocardial ischemia reperfusion in the rat (Squadrito *et al*, 1997a). Squadrito *et al*, demonstrated that chronic oestrogen treatment in the rat reduced expression of TNF-α and attenuated the expression of ICAM-1 and leukocyte infiltration. This resulted in a reduction in myocardial necrosis (Squadrito *et al*, 1997a, b). Although it is clear from these studies that oestrogen is cardioprotective, the differences in the effect on the cardiovascular system observed in clinical trials after one year compared with those after a longer follow up period were also reported in a study by Smith *et al* (2000). In this study, it was observed that during the acute period following surgery to induce MI, a greater mortality was observed in female rats treated with oestrogen and that these animals had an increased infarct size relative to placebo. However, in those animals that survived, chronic oestrogen treatment attenuated left ventricular dilation and normalised wall tension. It was suggested that this benefit may be attributed to the effect of oestrogen in the vasculature to reduce wall stress by reducing pre- and after- load. It was also suggested in this study that oestrogen may act directly on the myocardium by inhibiting the hypertrophic action of the endothelin system (Smith *et al*, 2000).

As a consequence of MI the heart may become hypertrophic. Initially this is a compensatory mechanism for the reduction in cardiac function due to the infarcted area. However, it eventually led to heart failure due to the reduction in left ventricular systolic function as indicated by a reduction in cardiac output. Chronic oestrogen treatment has been reported to reduce the development hypertrophy in a pressure overload model (van Eickels *et al*, 2001). In this particular study, it was reported that chronic supplementation of mice with oestrogen led to a reduction in hypertrophy which was attributed to an increase in the expression of atrial natriuretic peptide (ANP) and not to an inhibitory effect of oestrogen on the renin angiotensin system as a result of a reduction in AT_1 receptor expression. ANP is suggested to have anti-hypertrophic properties by attenuating protein synthesis via cGMP (Horio *et al*, 2000). This was confirmed by the finding that oestrogen treatment of female rats led to an increase expression of ANP (Jankowski *et al*, 2001). In addition to

inducing the increase in ANP levels, oestradiol reportedly induced bradycardia, the resulting reduction in oxygen consumption may have had an additional cardioprotective effect to that of the antihypertrophic effects of oestrogen (Jankowski *et al*, 2001). Interestingly, this study reported that ERα expression in the heart was greater than that of ERß and secondly, that the expression of ANP in the heart reflected that of ERα. This suggests that ERα may mediate the oestrogen induced increase in ANP.

1.4.3 Effects of oestrogen on the vasculature and the role of ERs

1.4.3.1 Effects of oestrogen on the vasculature

In addition to the effects of oestrogen on lipid metabolism, by which oestrogen may provide protection against CHD, other factors to be considered are the direct effects of oestrogen on blood vessels. Oestrogen replacement therapy is reported to decrease peripheral vascular tone in both healthy women and those with cardiovascular risk factors (Lieberman *et al*, 1995), and the vessel wall is now well recognised as an important target for oestrogen. Evidence is accumulating that oestrogen can act on the vessel wall to inhibit vascular smooth muscle cell proliferation (Chen *et al*, 1996) as well as influencing endothelial function (reviewed in Mendelsohn, 2002). Oestrogen may influence endothelial function by modulating one, or all, of the three main relaxing factors derived from the endothelium. These are NO, prostanoids and endothelium derived hyperpolarising factor (EDHF). Animal studies have largely concluded that chronic oestrogen treatment increases the basal release of NO from the endothelium (Hayashi *et al*, 1992, Paredes- Carbajal *et al*, 1995, Wu *et al*, 2000, Gonzales *et al*, 2001). However, multiple mechanisms may be involved in the oestrogen upregulation of NO mediated responses. Possible mechanisms include an increase in the amount of NOS protein, in the availability of substrate and/ or an increase in the levels of bioactive NO. The findings by Huang *et al* and Mac Ritchie *et al*, support an ability of oestrogen to upregulate transcription of the eNOS gene and protein expression and increased eNOS activity (MacRitchie *et al*, 1997, Huang

et al, 2000). Oestrogen may also stabilise eNOS mRNA and thereby increase eNOS protein expression (Sumi *et al*, 2001) and also reduce the levels of the endogenous nitric oxide synthase inhibitor, asymmetric dimethylarginine (ADMA, Dai *et al*, 2004). Taken together these changes would result in an increase in the bioavailability of NO. These effects are in addition to the antioxidant effects of oestrogen where oestrogen has been reported to downregulate the expression of NADPH oxidase in human endothelial cells and subsequently improve the NO/ O_2^- (superoxide) balance (Wagner *et al*, 2001).

Interestingly, chronic oestrogen treatment was also reported to increase the release of cyclooxygenase dependent vasocontricting metabolites of arachidonic acid (Paredes-Carbajal, *et al* 1995 and Miller and Vanhoutte, 1990). However, the effects of oestrogen on arachidonic acid metabolism are somewhat conflicting, as other studies have reported that oestrogen enhances the release of prostacyclin and subsequently vasodilation (Ospina *et al*, 2002, Ospina *et al*, 2003). It appears therefore, that the effects of oestrogen on the vasculature can be both species dependent and also dependent on the vascular bed studied.

In addition to NO and the prostanoids, a role for EDHF in mediating the endothelium dependent response to oestrogen has been reported (Dalle Lucca *et al*, 2000 and Liu *et al*, 2001). Liu *et al* reported that both normally cycling female rats in dioestrous (short term oestrogen deprivation) and those which were ovariectomised (long term deprivation), had a reduction in the endothelium-dependent relaxation response to ACh. This was attributed to the reduction in the release of EDHF when compared to rats which had elevated oestrogens (eg oestrous or supplemented with oestrogen following ovariectomy).

However, oestrogen may also regulate vascular tone by acting directly on vascular smooth muscle cells, independently of the endothelium. The enhanced contractile responses following oestrogen deprivation in previous models have been attributed to the reduction in endothelium-derived relaxing factors. However, whether the reduced contractile response is agonist-dependent or not is unclear. Similar to the finding by

Miller and Vanhoutte (1990), who studied rabbit aortas, Zhang and Davidge (1999), found that oestrogen deprivation of female rats had no effect on the contractile response of the mesenteric artery to the thromboxane analogue U46619. However, they did report that chronic oestrogen treatment of female rats, did reduce the response to PE and that this was attributed specifically to the reduction in the expression of α_1- adrenoceptors. This was the first study to show that oestrogen attenuated the contractile response by altering the expression of a specific receptor. Other studies have shown that the vasorelaxant effects of oestrogen on vascular smooth muscle cells have been independent of the agonist used to stimulate contraction.

In the rat aorta it was shown that oestrogen induced vasodilation that was blocked not only by L-NAME, which supports the role of oestrogen to stimulate the synthesis and subsequent release of NO from the endothelium, but also by tetraethyl ammonium (TEA) which is a K^+ channel blocker. Therefore, it was suggested that the vasorelaxant effects of oestrogen can be endothelium independent due to the modification of potassium (K^+) channel function (Abou-Mohamed *et al*, 2003). The calcium and voltage dependent K^+ channel, known as the maxi K^+ channel, is composed of two units, a pore forming alpha-unit and an accessory subunit, the ß1 subunit. The latter increases the channels sensitivity to both calcium and voltage and the activity of the ß1 subunit was enhanced by direct interaction with oestrogen (Valverde *et al*, 1999). This effect of oestrogen was shown to be independent of intracellular signals and was also induced by oestrogen which was conjugated to a carrier protein which was therefore unable to cross the cell membrane. However, later studies by Benkusky *et al*, demonstrated that oestrogen treatment increased the sensitivity of the maxi K^+ channel by increasing the transcription of the ß1 subunit of the maxi K^+ channel and only in vessels from animals that had been chronically treated with oestrogen (2002). Although there exists some discrepancy as to the mechanism by which oestrogen enhances the sensitivity of the K^+ channel, the resultant effect is that oestrogen increases K^+ efflux, repolarisation of the vascular smooth muscle cell and closing of calcium channels and subsequent relaxation. Oestrogen may also mediate relaxation in an endothelium-independent manner by

blocking Ca^{2+} channels of vascular smooth muscle cells (Salom *et al,* 2002). Using electrophysiological techniques on cultured, rat, aortic smooth muscle cells, oestradiol was shown to inhibit the barium inward current through voltage-dependent Ca^{2+} channels (Ogata *et al*, 1996) and to reduce the contractile response to noradrenaline by reducing calcium entry (reviewed in Tostes *et al*, 2003). However, the concentrations of oestrogen used in these studies were supraphysiological and therefore a direct effect of oestrogen on calcium channels is difficult to interpret as regards the effects of physiological concentrations of oestrogen.

1.4.3.2 The role of ERs

Two main areas of research on the effect of oestrogen on the vasculature have emerged from the literature, namely; vascular injury and vascular tone.

The model used most consistently in studies designed to induce vascular injury was balloon injury of the carotid artery. A study using female, ovariectomised rats administered either oestrogen or placebo (Bakir *et al*, 2000) reported that oestrogen attenuated the development of neointima formation following balloon injury of the carotid artery. This study also showed that the protection provided by oestrogen was receptor mediated as the antagonist blunted the effect of oestrogen in a dose-dependent manner. Although the ER antagonist used in this study would block both ERα and ERß a study published by Lindner *et al* (1998) suggested that ERß may have a role in the direct vascular effects of oestrogen as regards protection following vascular injury as the expression of ERß mRNA was found to have increased in human male blood vessels following vascular injury.

Due to the high degree of homology between the ERα and ß (reviewed in Nilsson *et al*, 2001) and the lack of commercially available receptor selective agonists and antagonists it has been difficult to study the individual effect of the receptors in vascular function. However, the generation of ERα (ERKO, Lubhan *et al*, 1993) and ERß (ßERKO, Krege *et al*, 1998) gene-modified mice have provided a useful tool for such studies.

It was believed that, by conducting studies with vascular injury models the role(s) of the individual ERs in mediating the protective effect of oestrogen would be elucidated. Iafrati *et al* reported, that oestrogen treated mice were protected against the increase in vascular medial area and smooth muscle cell proliferation, which developed following carotid arterial injury in placebo treated mice (1997). To determine whether oestrogen mediated its effect via ERα the same study was carried out in WT and ERKO mice and it was reported that both groups of mice were protected to the same degree against injury, by the administration of oestrogen. This suggested that oestrogen inhibited the increase in medial size and smooth muscle proliferation by a mechanism independent of ERα, possibly via ERß. However, subsequent studies of vascular injury in ßERKO mice (Karas *et al*, 1999) demonstrated that oestrogen treatment also protected against vascular injury in mice lacking functional ERß. One possible explanation for the protection observed in both the ERKO and ßERKO mice is that oestrogen may be able to act through either of the two ERs to provide the same degree of protection. It is also possible that in the knock-outs the expression of the remaining ER subtype is increased to compensate for the loss of the other receptor and therefore the expression of one ER is sufficient to provide protection. The generation of oestrogen receptor selective agonists or antagonists would be useful to either selectively stimulate or block the activity of one receptor in a WT animal whose expression of ERα/ ß has been unaltered. Interestingly, studies in DERKO (ERKO/ ßERKO double knock-out) mice found that they were still partially protected from vascular injury (Karas *et al*, 2002). This suggests that a component of protection provided by oestrogen to vascular injury is not receptor dependent, that a third oestrogen receptor exists or, as recently reported, that the ERKO mouse has a degree of residual activity (Couse *et al*, 1995) which is sufficient to provide protection.

Oestrogen has also been shown to have a regulatory role in vascular tone (Paredes-Carbajal *et al*, 1995, Hayashi *et al*, 1992, Wu *et al*, 2000) and chronic oestrogen treatment increases release of endothelium derived relaxing factors, *Section 1.4.1.2.2.* However, the role of the individual receptors in regulating vascular tone has only recently begun to be studied and the use of ERKO and ßERKO mice has

provided some interesting insights. Rubanyi *et al* reported that in male mice the basal release of NO was reduced in aortas from ERKO mice when compared to those from male WT controls (1997). It was shown that there was no difference in the stimulated release of NO, suggesting that the significant increase in cytosolic free calcium level and subsequent activation of NOS and release of NO may override the mechanism for the basal release of NO and the differences which existed between these groups of basal release. Although the role of individual ERs in producing the genomic effects of oestrogen in the vasculature have been poorly investigated significant attention has been spent on investigating the role of ERs on the rapid, nongenomic effects. For example, in human endothelial cells, incubation with a cell impermeant form of oestrogen was reported to rapidly increase NO production (Haynes *et al*, 2000a). As the rapid effect of oestrogen was blocked by either the non-specific ER antagonist, ICI 182,780 or an inhibitor of PI3-kinase, it was suggested that the non-genomic increase in NO produced by oestrogen was mediated by the PI3- kinase pathway and was ER dependent. Several studies have reported a role for ERα in mediating the rapid effects of oestrogen on the NO pathway in cultured cells. ERα has been shown to localise in signalling domains called caveolae, which are rich in many signalling molecules and activate eNOS (Chambliss *et al*, 2000). The activation of eNOS by oestrogen via the interaction of ERα with the PI3-kinase pathway, was reported to be physiologically relevant in the protection provided by oestrogen to ischaemia reperfusion injury in mice (Simoncini 2000). It was reported that mice treated with oestrogen had an increase in eNOS expression and a reduction vascular leukocyte accumulation that was attenuated by either inhibitors of PI3-kinase or NOS. However, this group did not address the presence and possible role of ERß nor confirm that this effect *in vivo* was ER mediated.

It was not known at the time of the study by Rubanyi *et al* that two ER subtypes existed and therefore they did not address the possibility that ERß may influence vascular tone (Rubanyi *et al*, 1997). It is only recently that characterisation of the role of ERß in the vasculature has begun. Nilsson *et al* (2000) reported that the relaxant response seen in preconstricted mouse aortas following acute administration of 17ß-oestradiol was greater in ßERKO mice than WT controls. This suggested that

ERß negatively regulated the release of NO, the opposite effect to that proposed for ERα. The difficulty in determining the role of ERß in the vasculature is that comparisons are being drawn between studies that are quite different. It is not possible to determine the role of ERs when comparing observations where oestrogen is being applied chronically within the physiological range to those in which it is being applied acutely at pharmacological concentrations. Indeed, we know from previous studies that physiological concentrations of oestrogen increase NO release whereas supraphysiological doses inhibit NO release and increase the release of constrictor prostanoids (Bolego *et al*, 1997). Therefore, it is conceivable that the observations of Nilsson *et al*, that ERß attenuates the release of basal NO, may be an artefact due to the high concentrations of oestrogen used.

1.4.4 Influence of oestrogen on atherosclerosis and regulation of blood pressure

1.4.4.1 Influence of oestrogen on atherosclerosis

Atherosclerosis is a complex inflammatory process which is characterised by the accumulation of lipid, macrophages and smooth muscle cells in intimal plaques. The endothelium is not only a physical barrier, protecting against blood clotting by forming a protective layer between underlying vascular smooth muscle cells and blood, but also mediates vascular tone. Endothelial dysfunction or injury is believed to be an early trigger of atherosclerosis. Indeed impaired NO release in endothelial dysfunction may contribute to the progression of the disease as NO, in addition to its vasorelaxant effects is involved in endothelial regeneration, reduction in platelet adhesion and inhibits leukocyte chemotaxis (Barbato *et al*, 2004). The pathogenesis of endothelial dysfunction results in an overproduction of reactive oxygen species (ROS) (reviewed in Yokoyama, 2004). In particular, the upregulation of NADPH oxidase shifts the NO/ O_2^- balance in favour of O_2^- and is closely associated with atherosclerotic progression. A strong relationship exists between hypercholesterolaemia and initiation of atherosclerosis (Gould *et al*, 1998) as

demonstrated by the clinical benefits of statins which reduce cholesterol. Due to the increase in oxidative stress native LDL, which is unable to penetrate the endothelium, is oxidised and can cross the endothelium by binding to scavenger receptors. Once in the subendothelial layer oxLDL can be taken up into macrophages to form foam cells which form the early stages of plaque development. Furthermore, oxLDL acts as a chemotactic factor for inflammatory cells, thereby increasing the inflammatory aspect of the process, which cause proliferation of vascular smooth muscle cells by releasing cytokines and growth factors. Collagen is produced in larger quantities by the smooth muscle and contributes to the development of a fibrolipid plaque.

The beneficial effects of oestrogen on lipid metabolism in addition to the positive influence on the NO pathway and the antioxidant effects of oestrogen as described in *Section 1.4.3*, suggested that oestrogen may be protective against atherosclerotic disease. Despite the described beneficial effects of oestrogen on lipid profile, the atheroprotective effects of oestrogen were suggested to be mediated by direct effects of oestrogen on the vascular wall as protection occurred at doses of oestrogen which did not alter lipid profile (Chae *et al*, 1997).

The role of oestrogen receptors in mediating the protection provided by oestrogen was suggested by the finding of Losordo *et al*, which reported that coronary arteries from premenopausal women with atherosclerosis were less likely to stain positive for ERs than vessels from women which lacked such lesions (Losordo *et al*, 1994). Methylation of the ER gene promoter inhibits expression of ER (Ottaviano *et al*, 1994). At this time, a second ER had not as yet been identified and therefore no distinction in ER subtype expression was made in these studies. However, a study by Post *et al* (1999), that followed on from Losordo *et al*'s early observation suggested a role for ERα. They reported that in the atherosclerotic region of a coronary artery ERα was reduced in comparison to expression in a non- atherosclerotic region and that the reduction in expression was due to methylation of the ERα gene promoter (Post *et al*, 1999).

Mice that do not express apolipoprotein E (Apo E-/-), which mediates the binding of lipid particles to lipoprotein receptors for the utilisation and redistribution of lipids (Pitas *et al*, 1987) suffer from premature atherosclerosis (Osada *et al*, 2000). These mice provide a good model for studying the influence of oestrogen in a model of hypercholesterolemia associated atherosclerosis. In such a model, oestrogen was reported to reduce lesion size and complexity, as determined by the presence of foam cells, formed/ developed fibrous cap and extent of calcification of the plaque, in female ApoE-/- mice (Hodgin *et al* 2001). By crossing the ApoE-/- mouse with the ERKO mouse, this group were able to demonstrate that the loss of ERα inhibited the reduction in lesion size but complexity of plaque was unaffected. This suggests that ERα mediates a component of the atheroprotective effect of oestrogen. However, a role for ERβ remains to be elucidated. As previously described in *section 1.4.3.2,* oestrogen has been shown to inhibit vascular smooth muscle cell proliferation and therefore this may be another mechanism by which oestrogen can protect against atherosclerosis. However, the mechanism by which oestrogen inhibits smooth muscle proliferation remains unclear.

1.4.4.2 Influence of oestrogen on blood pressure

A small cross over clinical trial revealed a small but significant decrease in both systolic and diastolic blood pressure that was due to a fall in peripheral vascular resistance (Luotola, 1983). However, clinical studies have provided confounding evidence that HRT either had no effect on blood pressure (Stassen et al, 1989) or as evident from a more recent study, that women receiving HRT had a smaller increase in blood pressure than those which were not (Mohle- Boetani *et al*, 2001). Furthermore, it cannot be ignored that the side effects of oral contraceptives is an increase in blood pressure (Nichols *et al*, 1993, Qifang *et al*, 1994). This increase in blood pressure observed with the use of oral contraceptives was attributed to activation of the renin angiotensin system as plasma renin activity was reportedly increased in users (Goldhaber *et al*, 1984).

Following the initial observations of oestrogen on blood pressure, clinical trials have tried to address the mechanisms by which oestrogen prevents the increase in blood pressure following the menopause. Blood pressure is dependent on cardiac output and peripheral resistance and can be regulated by the renin angiotensin system (RAS), renal function and by vascular function. Therefore, investigation has begun to characterise the effect of oestrogen on these systems.

1.4.4.2.1 Renin angiotensin system

Renin is secreted by the kidney and is the enzyme involved in the conversion of angiotensinogen, which is released from the liver, to angiotensin I. Angiotensin I is then converted to angiotensin II by angiotensin converting enzyme (ACE). Angiotensin II is a potent vasoconstrictor but also stimulates the release of aldosterone from the adrenal cortex. Aldosterone can increase blood pressure by acting on the kidneys to increase sodium reabsorption.

Literature suggests that oestrogen acts on elements of the RAS which would result in opposing outcomes. Studies have suggested that the anti-hypertensive effects of oestrogen may be mediated by inhibiting the RAS. Plasma renin activity is reported to be higher in postmenopausal women than premenopausal women and that premenopausal women have lower renin activity than age- matched men (Schunkert *et al*, 1997). Proudler *et al* and Gallagher *et al*, reported that oestrogen treatment reduced the activity of ACE in both human and rat models (Proudler *et al*, 1995, Gallagher *et al*, 1999). Following ERT, ACE activity was reduced by 20% in comparison to baseline values in postmenopausal women (Proudler *et al*, 1995). These findings suggest that the oestrogen induced reduction in blood pressure and shift of the hypertensive effects of RAS, induced by angiotensin II, to the vasodilatory actions of angiotensin I in both normotensive and transgenic hypertensive rats (Brosnihan *et al*, 1996, Brosnihan *et al*, 1997) may be due to a reduction in ACE activity. Oestrogen was also reported to act further downstream in the RAS pathway whereby it attenuated angiotensin induced aldosterone secretion in female rats (Roesch *et al*, 2000). However, contrary to these findings oestrogen

could act through the RAS to increase blood pressure. The promoter region of the angiotensinogen gene was reported to express an ERE (Feldmer *et al*, 1991) chronic oestrogen treatment of female rats increased serum levels of angiotensinogen, with a concomitant increase in expression of ERα in the liver, (Stavreus-Evers *et al*, 2001). However, caution should be applied when considering the physiological implications of such a finding as these effects were only observed with supraphysiological concentrations of oestrogen.

1.4.4.2.2. Renal function

The importance of the kidney in the regulation of blood pressure and in particular in hypertension is clearly demonstrated by the early findings of Curtis *et al*, who reported that transplantation of a kidney from a normotensive donor to a hypertensive recipient results in a reduction in blood pressure of the recipient to that within the normal range (Curtis *et al*, 1983). The kidneys play a critical role in the regulation of blood pressure by keeping constant the volume and composition of body fluids despite fluctuations in water and solute intake. One defect that can occur in hypertension is an alteration in the pressure natriuresis relationship, this refers to an increase in sodium excretion in response to an increase in arterial pressure (Hall *et al*, 1980).

At the time when this thesis was undertaken, few studies had been published describing an influence of oestrogen on renal function. In a subpopulation of postmenopausal Japanese women, oestrogen deprivation led to increased salt sensitivity and hypertension (Tominaga *et al*, 1991). This suggests that the anti-hypertensive effects of oestrogen may in part be mediated by improved renal function.

1.4.2.2.3 Vasculature

As described in *Section 1.4.3,* oestrogen has positive effects on the release of endothelium derived relaxant factors and may also reduce contractile tone of vascular smooth muscle cells in an ER dependent manner. However, whether the effect of oestrogen on the vasculature can modulate blood pressure was not

determined in those same studies and has been an area of oestrogen research in the cardiovascular system that has been poorly studied to date.

Initial studies in the literature reported that the beneficial effects of oestrogen on the bioavailability of NO may influence blood pressure. Doursout *et al* demonstrated that oestrogen deprivation of female rats reduced the pressor response induced by inhibition of NOS however, baseline blood pressure was unchanged (2002). However, few reports have been published investigating the influence of oestrogen on the vasculature and how this may affect blood pressure.

Clinical observations have suggested that ERβ may mediate the anti-hypertensive effects of oestrogen as a link was reported in Japanese women between ERβ polymorphisms and hypertension (Ogawa *et al*, 2000). However, further investigations into the mechanisms by which oestrogen may affect blood pressure and the ER involved have not as yet been published.

1.5 General aims of the study

Oestrogen was considered to have beneficial effects on the cardiovascular system as initial clinical observations suggested that oestrogen lowered blood pressure (Strassen *et al*, 1989, Seely *et al*, 1999) and reduced the incidence of coronary heart disease (Sullivan *et al*, 1990). However, more recent controlled clinical trials have reported no overall benefit of HRT in the prevention of CHD (Hulley *et al,* 1998, Cherry *et al*, 2002) and therefore cast some doubt over the use of oestrogen in the prevention of cardiovascular disease. Following from the initial observations of the beneficial effects of oestrogen in the cardiovascular system, animal studies reported that oestrogen may provide protection against MI due to the antihypertrophic properties of oestrogen in a rat model of MI (Jankowski *et al*, 2001). In the vasculature oestrogen was reported to regulate vascular tone by enhancing the release of endothelium-derived vasodilators such as NO (Hayashi *et al,* 1992, Wu *et al,* 2000), prostacyclin (Ospina *et al,* 2002) and EDHF (Liu *et al,* 2001). However,

only recently have the involvement of specific ERs in mediating these effects been studied. The generation of ERKO mice demonstrated that oestrogen acting through ER may increase the bioavailability of NO and subsequently enhance vasodilation (Rubanyi *et al,* 1997). However, literature has not yet fully characterised the expression of ERs in the animal models studied, nor investigated whether the effects of oestrogen on the vasculature may also be mediated by ERβ. Furthermore, whether the vascular effects of oestrogen mediated via ERs, contributes to the regulation of blood pressure has currently not been determined.

The main aims of this thesis were to determine, using a mouse model whether oestrogen acting through ERβ modifies vascular function and contributes to the regulation of blood pressure. This was investigated by firstly, determining the distribution and cellular localisation of ERα and ERß in the cardiovascular system and whether this was altered under pathophysiological conditions such as myocardial infarction and atherosclerosis. Once expression of ERs in the mouse cardiovascular system had been determined, the effect of endogenous oestrogen, in male mice, on the vascular system and the role of ERß using the βERKO mouse. Finally, our aim was to determine the effect of oestrogen status, and the role of ERß, on vascular function and whether this contributed to the regulation of blood pressure in mediating these effects. This was achieved by studying female mice of different oestrogen status and through the use of the novel ERß antagonist, Org 44488, in preference to the ßERKO mouse.

Chapter 2

Methods

2. Methods

All experiments involving animals were conducted in accordance with Home Office guidelines as outlined in the Animals (Scientific Procedures) Act 1988 and under the terms of Project Licence number 60/2813.

2.1 Animals

Animals were housed in a controlled environment on a 12hr light- dark cycle (lights on 0700 – 1900hrs) and a set climate 21- 22°C with free access to water and standard mouse chow containing 75.09% carbohydrate, 14.38% protein, 2.71% oil and 0.25% salt (Special Diet Services (SDS), Witham, Essex, UK).

Animals used were, oestrogen receptor ß knock-out (ßERKO), ApoE knock-out (-/-) and C57Bl6 mice (Harlan Orlac, Bicester, Oxon, UK).

ßERKO mice generated by a targeted disruption of exon three of the ERß gene as previously described (Krege *et al.* 1998), were obtained from the *Department of Medical Nutrition, Karolinska Institute, Sweden*. The colony was established in our laboratory by breeding ERß heterozygous mice to yield ßERKO (-/-) and wild-type (WT, +/+). Both WT and ßERKO mice were on a mixed C57Bl6/SvJ129s genetic background.

ApoE-/- mice on a C57Bl6 genetic background, were kindly made available by Dr I. Megson who has a colony established at the University of Edinburgh.

Mice were allowed an acclimatisation period of a week in their new environment prior to any surgical or experimental procedures.

2.2 Genetic and Phenotypic analysis of ßERKO mice

2.2.1 Genotyping of the ßERKO colony

The genotype of the mice was determined by polymerase chain reaction (PCR) which was used to identify WT and ERß targeted alleles.

2.2.1.1 DNA extraction from tail biopsies

Tail biopsies were taken from mice following weaning, aged 3-5 weeks under halothane anaesthesia. Tail biopsies, 0.5cm in length from the tip of the tail, were digested overnight at 55°C in tail buffer (1.0M Tris- HCl; pH8, 0.5M EDTA; pH8, 1.0M NaCl, 10% SDS, dH_2O) containing $10mgml^{-1}$ Proteinase K (Promega, Southampton, UK).

2.2.1.1.1 Method 1

Digested tails were incubated with 20µl of $20\mu g\mu l^{-1}$ RNAse (Promega, Southampton, UK) for 1hr at 37°C following which 6µl mercaptoethanol and 600µl Tris washed phenol (Fisher Scientific, Loughborough, UK) was added to each tube and placed on a vertical rotator for 15mins. The samples were centrifuged at 13,000rpm for 2mins and the aqueous phase and interphase were transferred to a fresh tube. 300µl of phenol and 300µl of 'chloroform' (chloroform: isoamyl alcohol 24: 1) were added to each tube and rotated for 5mins prior to centrifugation as above. The aqueous phase and interphase were transferred to a fresh tube, 600µl 'chloroform' and the sample was rotated for 5mins.

Following centrifugation as above, the aqueous phase was transferred to a fresh tube, 600µl isopropanol was added and the tube was inverted several times until DNA was precipitated out. To pellet the DNA, the sample was centrifuged at 13,000rpm for 2mins and the supernatant was removed. 180µl of 70% ethanol was added to the DNA pellet to remove any isopropanol, centrifuged for 2mins and the supernatant

removed. This step was repeated twice. Finally, the DNA pellet was dissolved in 200μl of TE buffer (10μM Tris, 1μM EDTA) and rotated for 1hr at 37°C.

2.2.1.1.2 Method 2

Similar to the phenol extraction protocol, tail tips were digested overnight and treated with RNase (Promega, Southampton, UK). However, following this the samples were placed on ice for 10mins or until a precipitate formed. Samples were centrifuged at 12,000 rpm for 2mins, placed on ice and the supernatant removed. An equal volume of isopropanol was added to the supernatant and rotated to aid precipitation of DNA before being centrifuged for 2mins at 12,000 rpm to pellet DNA. The supernatant was removed and the pellet was washed with 70% ethanol. Ethanol was removed and replaced by 200μl TE buffer. The samples were then placed at 94°C for 5mins to inactivate the Proteinase K (Promega, Southampton, UK) and subsequently 37°C for 30mins to dissolve the DNA pellet.

2.2.1.2 Polymerase Chain Reaction

Each PCR reaction (25μl) contained 1U *Taq* DNA polymerase (Biothermstar, Genecraft, Munster, Germany) 0.5mM dNTPs (Promega, Southampton, UK), 0.3μM specific primers (MWG Biotech) for gene of interest, *Table 2.1*, 10x PCR reaction buffer containing 1.5mM $MgCl_2$ (Biothermstar, Genecraft, Munster, Germany), and 1μl DNA. The samples were transferred directly from ice to the thermocycler where the following reaction conditions were applied:-

An initial denaturing step of the DNA was carried out at 94°C for 15mins. Amplification of DNA was as a result of 30 cycles of three defined stages. Firstly, the DNA was denatured (92°C for 30s), which was followed secondly by the annealing of primers to their complimentary DNA sequence (63°C for 30s) and thirdly an elongation stage, the primers were enzymatically extended (68°C for 2mins). Once the cycles were completed an additional elongation step of 68°C for 10mins was carried out.

2.2.1.3 Agarose gel electrophoresis

PCR products were fractionated through a 1% agarose gel (LE, Agarose Seakem, BMA, Rockland, USA) stained with ethidium bromide (0.04%, Sigma-Aldrich Company Ltd., Dorset, UK) which enabled PCR products to be visualised under UV fluorescence, *Figure 2.1*. 5µl of blue-orange 6x6 loading dye (Promega, Southampton, UK) was added to 25µl of sample of which 12µl was loaded into the gel well. A 100bp ladder (Promega, Southampton, UK) was run on all gels, the well containing the DNA ladder was loaded with a 6µl mixture composed of 4µl DNA ladder and 2µl loading dye.

The gel was run for 1hr 30mins at 100V following which the PCR products were visualised in an ultraviolet transilluminator, *Figure 2.1* (Ultraviolet Products Ltd., Cambridge, UK) attached to a video display unit (SONY Coorporation, Japan).

Table 2.1 ***PCR primer sequences and product size for the wild type and targeted ERβ allele***

Gene	Primer sequence	Product size
ER beta WT allele	5'-AGA ATG TTG CAC TGC CCC TGC TGC T-3' 5'-GGA GTA GAA ACA AGC AAT CCA GAC ATC-3'	650bp
ER beta targeted allele	5-AGA ATG TTG CAC TGC CCC TGC TGC T-3' 5'-GCA GCC TCT GTT CCA CAT ACA CTT C-3'	500bp

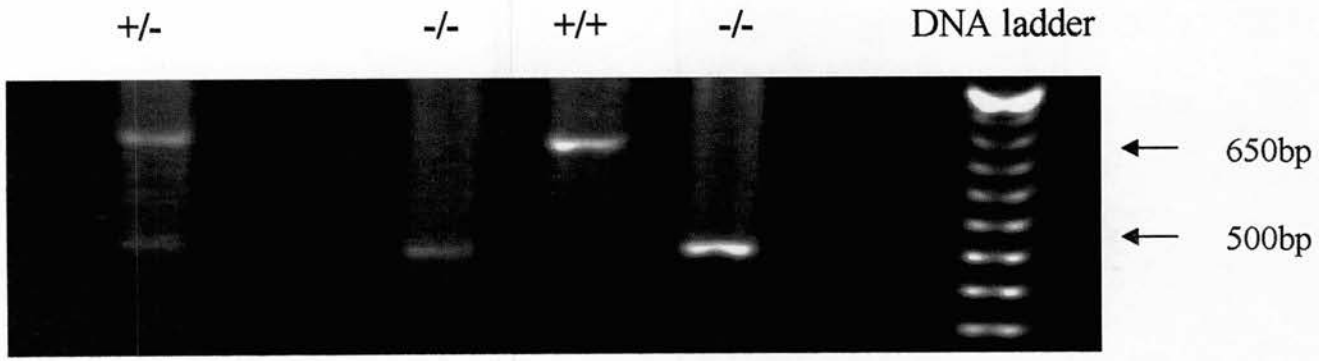

Figure 2.1 ***PCR for ERβ alleles.***
Gel electrophoresis of PCR products for WT +/+, heterozygous +/- and βERKO -/- mice. The arrowheads indicate 650bp product which was specific for the wild type allele and the 500bp product for the targeted insertion.

2.2.2 Phenotypic analysis of the ßERKO mouse

To confirm that the colony of ßERKO mice established in our lab were phenotypically identical to the colony described by Krege *et al* and housed in Sweden, we analysed their ovarian phenotype (Krege *et al*, 1998).

2.2.2.1. Harvesting of ovaries.

Ovaries were harvested from female WT and ßERKO littermates and processed as outlined in section 2.4.1

2.2.2.2. Histological analysis of ovarian phenotype

To determine whether genetic background affected ovarian morphology serial sections (6μm thick) were stained with hematoxylin and eosin. As sexually mature females contain a heterogeneous population of follicles at different developmental stages (Pederson and Peters, 1968, Butcher and Kirkpatric- Keller, 1984), an average of 30 sections were cut from each ovary and every fifth section was examined to obtain an overall view of the follicular populations. Briefly, sections of ovary were immersed in Harris' hematoxylin for 5 mins, washed in tap water, differentiated with acid alcohol for 5s and then washed in running tap water. Thereafter, the sections were immersed in eosin for 30s before being rinsed in tap water. The sections were rapidly dehydrated through increasing concentration of alcohol (70, 80, 95%) to absolute alcohol (2 x 1min), histoclear for 5 mins and then to xylene (2 x 5 mins). Finally, the slides were dried and coverslips mounted with DPX mounting medium (BDH Laboratory Supplies, Dorset, UK).

2.3 Surgical procedures

Prior to each surgical procedure animals were weighed. Anaesthesia was introduced either by inhalation of either halothane or isoflurane (for bilateral ovariectomy), or by i.p. injection of ketamine ($76mgkg^{-1}$)/medetomidine ($1mgkg^{-1}$) (for recovery radiotelemetry) or ketamine ($100mgkg^{-1}$)/xylazine ($10mgkg^{-1}$) (for coronary artery ligation, non-recovery blood pressure and echocardiography). Upon onset of anaesthesia, analgesia was administered in the form of buprenorphine ($0.05mgkg^{-1}$, s.c.).

Aseptic techniques were applied to all surgical proceedures. Hair was removed from the surgical site by clippers. Depilatory cream (sensitive hair remover, Nair, Carter-Wallace) was then applied to prevent hair from entering the incision site and swabbed with Vetsept® (Genusxpress, Aberdeen, UK). Cling film was draped over the animal leaving only the surgical site exposed. Lacrilube® (Genusxpress, Aberdeen, UK) lubricating ointment was applied to the eyes to prevent desiccation and the mouse was placed on a heating pad to maintain body temperature. The depth of anaesthesia was determined prior to, and at regular intervals throughout, surgery by pinching the foot of the mouse tightly and observing any changes in respiration.

Once surgery was completed, the anaesthetic effect of both ketamine mixtures was reversed by the α_2-adrenoceptor anatgonist atipamezole ($1mgkg^{-1}$, s.c.). Post-operatively, animals were monitored at least every 10min until ambulatory, in a recovery chamber set at an ambient temperature of 37°C containing mash and water. Animals were weighed and closely observed on the days following surgery for any signs of infection or poor health.

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2.3.1 Recovery surgical procedures

2.3.1.1 Regulation of and Staging of the oestrous cycle

2.3.1.1.1 Bilateral ovariectomy

One variable in doing studies in female animals is the varying levels of oestrogen during the oestrus cycle. This problem can be overcome by controlling the levels of circulating oestrogen by performing ovariectomy (ovx). This procedure involves the removal of the ovaries, which are the principal site of oestrogen production, and implantation of pellets that release oestrogen, to achieve physiological concentrations (0.05mg/pellet, 60 day release, 50-100pgml^{-1}, Innovative Research, Sarasota, USA), or placebo (60 day release, Innovative Research, Sarasota, USA). Female WT mice were randomised to receive either placebo pellets (-E) or oestrogen (E) containing pellets (+E,) to re-establish circulating physiological levels of oestrogen.

Levels of oestrogen are elevated during both the pre- and post- ovulatory stage of the oestrous cycle. Oestrogen stimulates the cells of the stratum basalis of the uterus to undergo mitosis and promotes vascularisation and thickening of the endometrium. When oestrogen levels are low, the endometrium is thin and atrophied as only the stratum basalis of the endometrium remains. Therefore, due to the proliferative role of oestrogen on the uterus, uterine weight can be used as an indication of the circulating levels of oestrogen and to confirm oestrogen status following ovx, *Figure 2.2* and *Figure 2.3.*

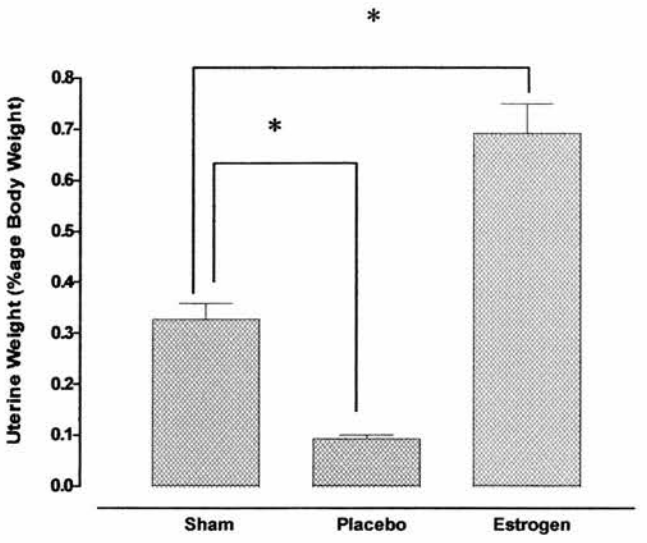


Figure 2.2 ***Effect of oestrogen status on uterine weight***

Uterine weight of sham ovx mice (n=15) and ovx mice implanted with either oestrogen 0.05mg/pellet (n=9) or placebo (n=8) pellets. Values are expressed as mean± SEM and analysed using a 1- way ANOVA followed by a Tukey post- hoc test, $p<0.05$

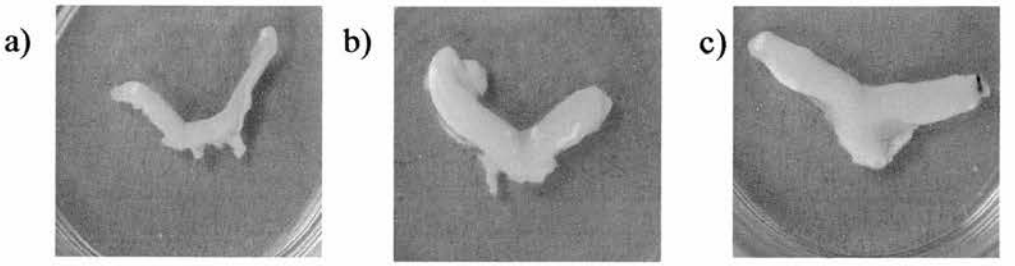


Figure 2.3 ***Uteri from mice of different oestrogen status***

Uteri were removed from mice that had undergone ovx and treated with a) placebo b) sham ovx and c) ovx and supplemented with oestrogen 0.05mg/pellet.

Anaesthesia was induced by placing the mouse in a chamber containing 4% halothane or isoflurane (Halothane-vet, Merial Animal Health Ltd., UK) vaporised in a mixture of O_2 (1.5l min^{-1}) and N_2O (0.5l min^{-1}) for halothane and O_2 alone for isoflurane. Anaesthesia under halothane and isoflurane (1.7-2% in gas mixture) was maintained via a nasal cone. The lumbar dorsum was shaved bilaterally and an incision in the dorsal flank, of about 5mm in length, penetrating the abdominal cavity was made. The parovarian fat pad was identified and retracted and the exposed ovary and associated oviduct were severed and removed. A ligature (5/0 Mersilk, Ethicon Ltd.) was secured around the severed ovarian vasculature to maintain haemostasis. The uterine horn was returned to the abdominal cavity and the incision was closed, firstly closing the abdominal wall (5/0 Mersilk, round bodied, Ethicon, UK) and then closing the skin (5/0 Mersilk, cutting, slim blade, Ethicon, UK). A small, subcutaneous incision was made inter scapular into which the pellet, either –E/ or +E was placed and the incision subsequently closed.

Once the animal was ambulant it was returned to a cage containing a maximum of three other animals, each of which had undergone the same surgical procedure. Food and water were freely available. Mice were housed with animals within the same oestrogen treatment as this prevents exposure to oestrogen via grooming of one another.

Where mice were undergoing a second surgical procedure, a period of 7 days was allowed for the animal to fully recover.

2.3.1.1.2 Staging the oestrous cycle

Vaginal smearing of experimental mice, in which the levels of oestrogen were not controlled by ovariectomy and the implantation of oestrogen/ placebo releasing pellets, was necessary to determine the ovarian cycle. The oestrous cycle was monitored for 4 consecutive days, 1000-1030hrs, prior to surgery or towards the end point of a study based on vaginal smear patterns and that ovulation occurs

approximately every 4 days. This was done either prior to surgery or towards the end point of a study.

The speculum was moistened by immersing in sterile saline prior to being introduced in the mouse' vagina. Once inside the cervix the speculum was rotated to ensure collection of cells and transferred to a glass slide and allowed to air dry. The slides were taken to water prior to staining with toluene blue for 3mins to allow easy visualisation of cell type, *Figure 2.4*. Smears were classified into one of 4 phases of the oestrous cycle: proestrous was characterised by a smear of leukocytes and elongated nucleated epithelial cells; large cornified epithelial cells were exclusively found in oestrous; metoestrous was characterised by a smear composed of equal numbers of nucleated epithelial cells and leukocytes; and a smear consisting almost exclusively of leukocytes depicted dioestrous (Allen 1922, Fox 1970)

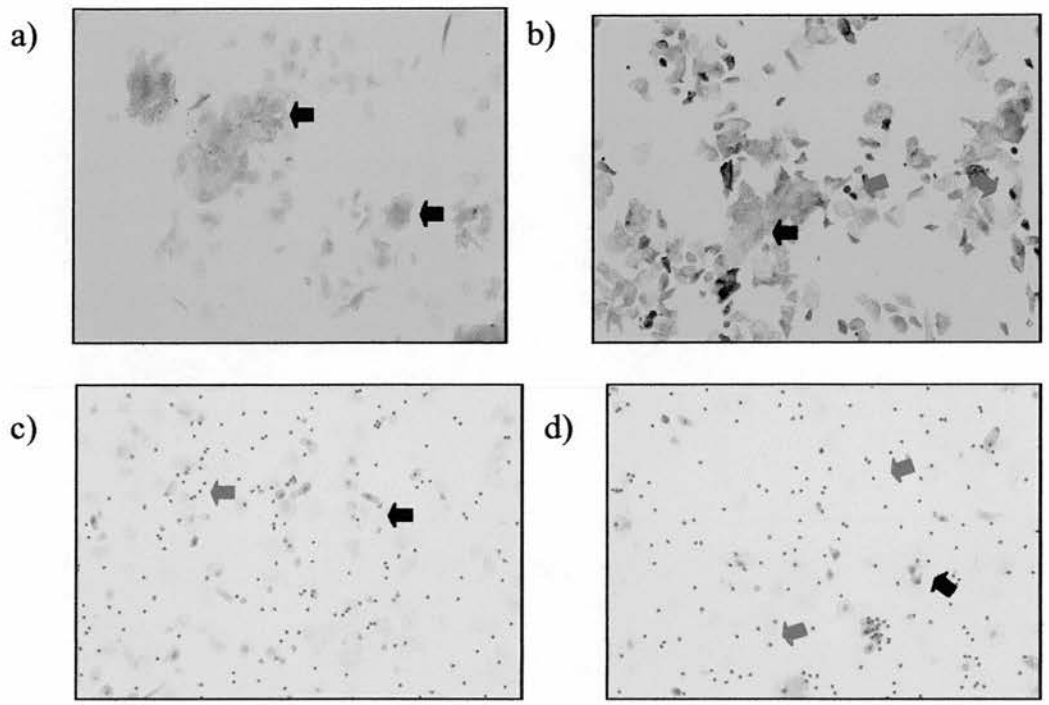


Figure 2.4 Vaginal smears from female mice during the oestrous cycle

The ovarian cycle of sham ovx female mice was determined by vaginal smearing and smears were classified into 4 stages of the oestrous cycle a) oestrous b) metoestrous c) dioestrous d) proestrous. Black arrows indicate epithelial cells and red arrows indicate leukocytes (x20 magnification).

2.3.1.2 Radiotelemetry

Implantable, miniaturised mouse blood pressure transmitters (Data Sciences International, PA11-C20) were used to directly measure arterial pressure, activity and heart rate digitally from within individual animals. These devices are currently thought to provide the most sensitive and accurate method to monitor blood pressure in conscious, free-moving mice (Mills *et al*, 2000). Radiofrequency signals from the transmitters are converted to serial bit streams and data are collected and stored to disk using the Dataquest A.R.T. data acquisition system (Data Sciences International, St Paul, MN).

Surgery was carried out with the assistance and subsequent supervision of Mrs G. Brooker. All animals were prepared for surgery as previously described and anaesthetised using an anaesthetic mix of ketamine/medetomidine. A ventral midline incision and a horizontal incision were made in the scapular region of the neck to allow implantation of the body of the device. A tunnel was formed from this incision across the left pectoral area and was enlarged using blunt scissors to form a pocket along the left flank and lubricated by 1ml of warmed sterile saline.

Once the pocket had been made the carotid artery was isolated using sterile saline soaked Q-tips to gently push the submandibular glands to the right and away from site of access. Once the thorax was visualised an entry site was made to the left of the thorax and between the sternohyoid and omohyoid muscles. The left common carotid artery was isolated using fine tipped vessel dilation forceps with great care so as to separate, whilst avoiding disturbing, the vagus nerve which runs parallel to the artery. The artery was ligated at the bifurcation into the external and internal carotid arteries and temporarily occluded at about 8-10mm lower with a second suture, *Figure 2.5a*. Traction was applied to the most distal ligature which was then taped into position putting tension on the artery. The most proximal ligature was elevated using Spencer Wells, which were used as a weight to apply traction. This occludes blood flow into the remaining middle portion of the artery which was then isolated so that the blood vessel could be incised and the catheter introduced. Using a bent-tipped 25G syringe needle as a catheter introducer. The carotid artery was punctured

at the level of the bifurcation and the catheter was inserted and pushed caudally into the vessel towards the occluding suture. The most proximal suture was then gently released and the catheter was further advanced into the vessel to the point at which the taper begins, about 13-14mm in length from tip, *Figure 2.5b.* At this point both the proximal and distal sutures (5/0 Mersilk, Ethicon, UK) were tied around the vessel and catheter and secured in place using Vetbond, *Figure 2.5c.*

Once the catheter had been secured in place, the body of the telemetry probe was introduced into the pocket along the left flank. Prior to inserting the body of the probe, the pocket was lubricated with 1ml warm sterile saline to allow easier insertion of the device. The body of the device was then pushed into the pocket between the front and rear limb and the incision site was closed (5/0 Mersilk, cutting, slim blade, Ethicon, UK), *Figure 2.5d.*

Once ambulant, the animal was placed in a cage containing food and water. Analgesia was made available as jelly (buprenorphine, 0.5mgkg-1), that was placed inside the cage enabling the animal to self medicate during recovery. Body temperature was maintained at 37°C by keeping the animal on a heating pad for a further 48hrs or until it was fully conscious and active which ever was later. To prevent dehydration, 1ml warm 0.9% sterile saline (s.c.) was administered at time of surgery and repeated after 12hrs if the animal appeared dehydrated. This was determined by checking the elasticity of the skin and extent of weight loss.

Animals were allowed a 10 day recovery period prior to commencing study, during which time preliminary tests were carried out to confirm that blood pressure and heart rate had stabilised. During the experimental period blood pressure, heart rate and locomotor activity were sampled for a period of 10s every 15mins over the entire experimental period (refer to *Figure 5.1*, *Chapter 5*).

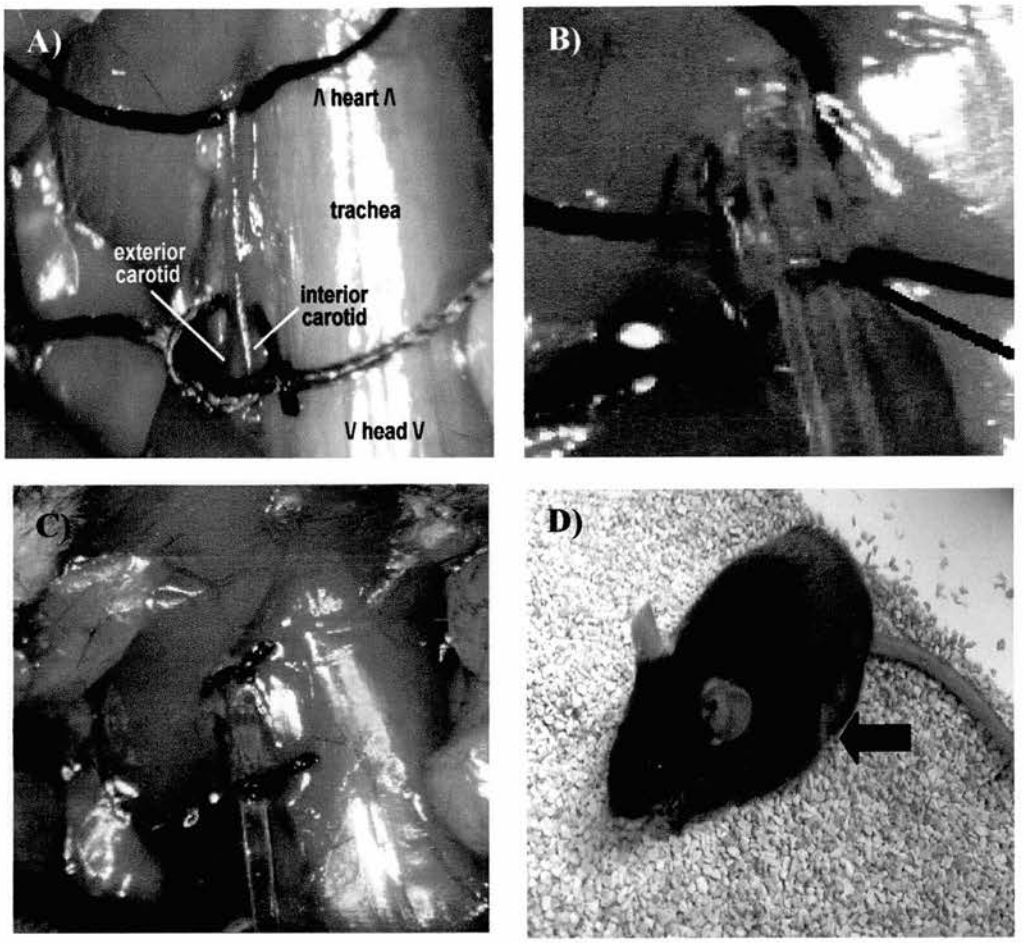


Figure 2.5 ***Telemetry implantation.***

A) Isolation of left common carotid artery B) Insertion of telemetry catheter into the left common carotid artery and progressed into artery until catheter notch is in line with the bifurcation C) Cathter is secured in place. D) Animal which has been implanted with telemetry device, block arrow indicates position of the body of the telemetry device on the left flank of the recovered mouse.

2.3.1.3 Coronary artery ligation

Dr I. Sharif performed the surgery for the induction of myocardial infarction by coronary artery ligation (CAL), *Figure 2.6.* In brief, C57BL6 female mice (14- 16wks) underwent a left intercostal thoracotomy to expose the heart. A ligature (6/0 prolene suture, Ethicon, UK) was placed around the left main coronary artery which was tied in CAL mice and pulled under the artery in sham-operated mice. The thorax was then closed using a silk braided suture (5/0 mersilk, Ethicon, UK). Body temperature was maintained at 37°C by being kept on a heating pad for a further 48hrs or until animal was fully conscious and active.

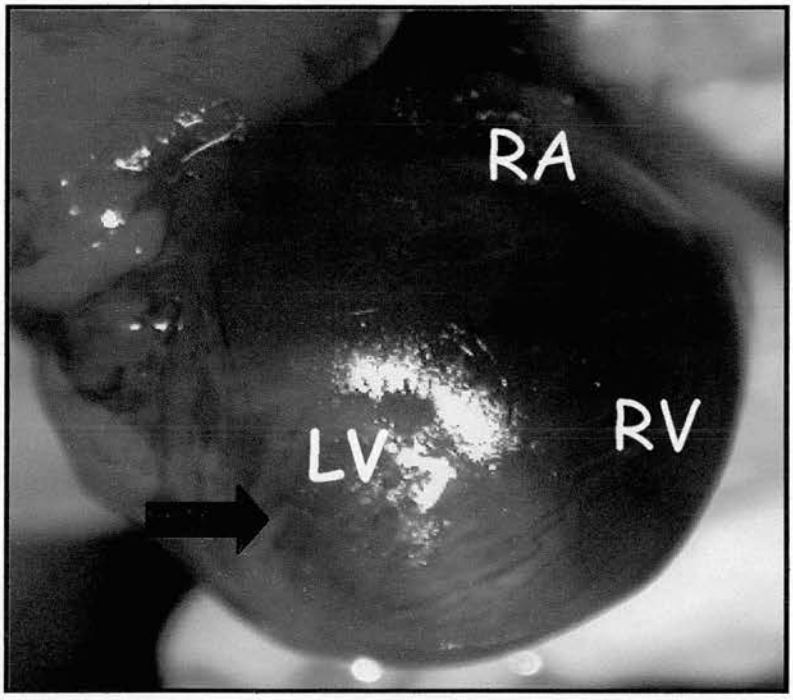


Figure 2.6 Image of a female mouse heart which had undergone CAL. Block arrow indicates area of infarct in the left ventricle.

2.3.2 Non- recovery surgical procedures

2.3.2.1. Measurement of blood pressure

Dr I. Sharif kindly performed surgical implantation of the Millar catheter. All animals were prepared for surgery as previously described. Once fully unconscious the right carotid artery was isolated and cannulated with a 1.4 Fr (0.46mm) high fidelity micromanometer catheter (Millar Instruments Inc., Houston, TX) connected to a control unit (TCB-500, Millar Instruments Inc.). After a stabilisation period of 10mins, measurements of arterial pressure and heart rate were obtained. All measurements were recorded on a MAC computer using a Power Lab system. The Miller control unit was connected to a data acquisition system which consisted of a Powerlab (Powerlab/8sp, AD Instruments), which was connected to a MAC computer with CHART v4.1 software (AD instruments).

2.3.2.2 Assesment of cardiac function

Echocardiography was kindly performed by Dr I. Sharif. To enable clear visualisation of the heart the mouse was placed in left lateral decubitus position and the chest area and probe head were covered with acoustic gel (Clear Image medical ultrasound couplant, Diagnostic Sonan Ltd., Livingston, UK). Care was taken when placing the probe on the left hemithorax in order to avoid excessive pressure which could have induced bradycardia. Cardiac ultrasound imaging was performed using a 128-element probe (10-22MHz) with DIASUS P5.26 software (Dynamic Imaging), the depth of which was set at 26mm with 1 focus line. Parasternal long- and short-axis views were obtained. 2-dimensional echocardiographic loops of at least 20 cardiac cycles, b-mode tracings were stored on cd for offline analysis.

To assess cardiac function, left ventricular (LV) diameters during end-diastole (LVEDD) and end-systole (LVESD) were measured to calculate percentage fractional shortening (%FS = 100((LVEDD - LVESD)/LVEDD). LV areas during end-diastole (LVED area) and end-systole (LVES area) were measured to calculate percentage ejection fraction (%EF = 100((LVEDA – LVESA)/LVEDA). All

measurements were calculated from images taken from the long axis view where images were considered adequate for measurement when >75% of the epicardial and endocardial contour could be adequately visualised.

2.4 Tissue collection for in- vitro purposes

2.4.1 Histology, immunocytochemistry and myography.

Organs were removed and then rinsed in ice-cold Krebs solution before undergoing a rough dissection to remove excessive fat or connective tissue. Hearts were cut from apex to base and all organs were blotted prior to being weighed

The thoracic aorta was removed and pinned out on a silicone-coated (Sylgard, Dow-Corning, UK) dissecting dish containing ice-cold Krebs-Henseleit solution (Krebs).

The mesenteric bed was exteriorised and the intestine feeding the vascular bed was removed to a sylgard plate containing ice-cold Krebs. Both the thoracic aorta and an area of the first order mesenteric artery (the area distal to the first branch point of a vessel leading on to the intestinal wall) were cleaned of connective tissue in preparation for mounting onto a myograph.

2.5 Pharmacological studies of isolated mouse blood vessels

2.5.1 Mounting of mouse blood vessels onto the myograph

2.5.1.1. Thoracic aorta

Following dissection, the aorta was cut into two transverse rings 4mm in length and mounted on a small vessel myograph (Model 700MO, Danish Myotech, Denmark) containing 10ml of oxygenated (95% O_2, 5% CO_2) Krebs at 37°C. Mouse aortic rings were placed under an optimum resting tension of 0.75g and allowed to equilibrate for 60 minutes, during which time the tissue was washed periodically with Krebs and the tension adjusted when necessary to 0.75g, equivalent to 7.36mN.

2.5.1.2 First order mesenteric artery

The vessel was prepared as previously described for mounting onto a wire myograph containing 10ml of oxygenated Krebs solution warmed to 37°C. Two isolated rings of first order mesenteric artery were cut to a length of 4mm of which 2mm constituted the area of the artery to be studied and the remaining 2mm of tissue used to feed the artery onto the wires which was removed once the vessel was mounted. Two tungsten wires (4μM diameter) were inserted through the lumen of the isolated artery (2mm in length). A force transducer connected to one of the two wires recorded mechanical activity, isometrically. The second wire was attached to a support carried by a micromanipulator. First order mouse mesenteric arteries were placed under an optimum resting tension of 0.2g, equivalent to 1.96mN. In a manner similar to the aorta set-up, mesenteric arteries were allowed to equilibrate for 60mins with tension being adjusted as necessary.

Isometric tension from the vessels was measured by a DSC6 strain gauge transducer, processed by a MacLab/4e analogue digital converter and displayed through Chart™ software.

All vascular studies performed on mouse mesenteric arteries were carried out by Dr P. Medina

2.5.2 Standard start protocol

After 60mins, the following protocol was carried out before commencing the experimental protocol in order to normalise contractile data:-

The maximum contractility of the tissue was determined using Kreb's containing 60mM KCl (equimolar replacement of NaCl by KCl) and the integrity of the endothelium assessed by measuring endothelium dependent relaxation to acetylcholine (ACh) of noradrenaline (NA) induced tone. Aortic rings were subjected to two exposures of 60mM KCl, followed by 60mM KCl combined with NA 10^{-7}M and a final exposure to 60mM KCl until the response had plateaued, separated by subsequent wash-out and a resting period of 10mins. Thereafter, the tissue was exposed twice to the adrenergic receptor agonist NA (10^{-7}M) and on the second exposure instead of wash-out the presence of endothelium was confirmed by examining the relaxant response to the endothelium dependent agonist ACh (10^{-6}M), *Figure 2.7.* Aortic rings with relaxations to ACh greater than 70% were considered as having an intact endothelium and used in the experiments. The pharmacological protocols which were applied following the stabilisation period and the above 'normalisation' protocol are described in the relevant sections of Chapters 4 and 5.

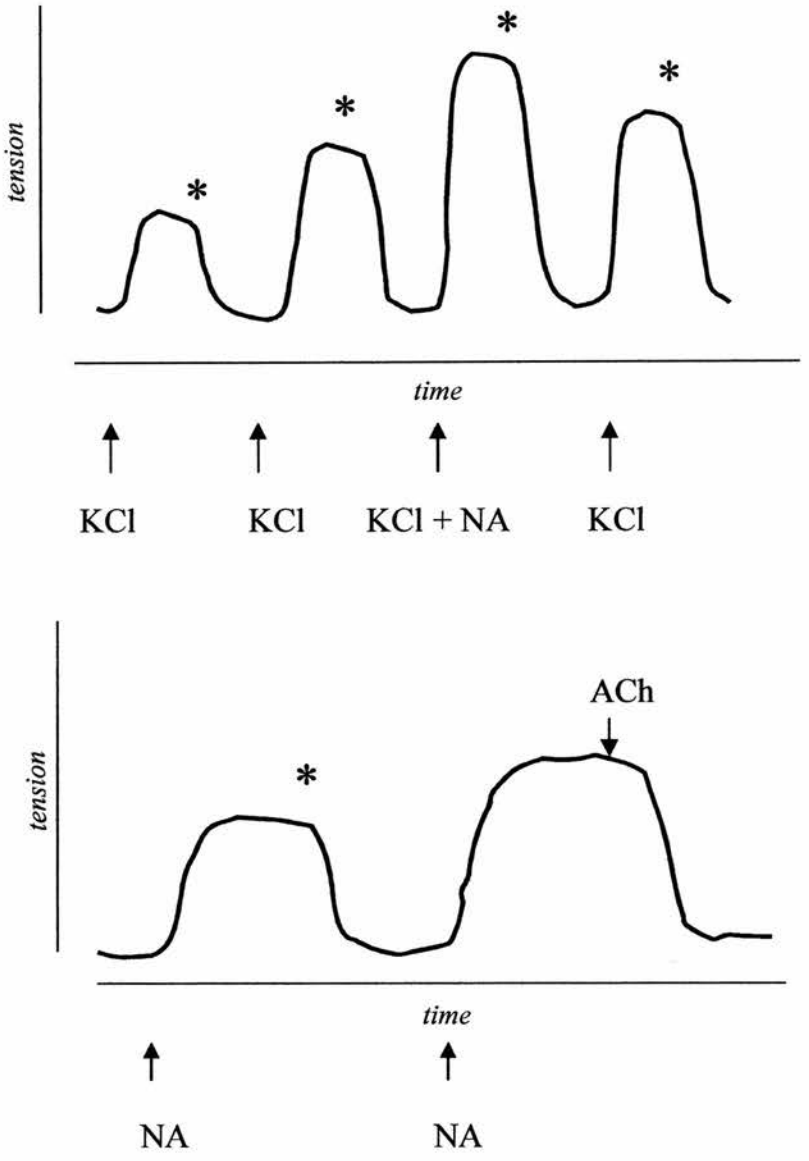


*Figure 2.7 Schematic diagram of the responses of aorta and first order mesenteric vessels from mice to the standard start protocol, KCl; 60mM, NA; 10^{-7}M, ACh; 10^{-6}M and * indicates wash out.*

2.6 Histology and immunocytochemistry

2.6.1 Processing of tissue

Following harvesting, *section 2.4.1,* tissues were fixed for 16hrs in 10% neutral buffered formalin. This noncoagulant fixative was chosen as it allows good histological detail of the tissues to be preserved because it does not drastically change the fine network of tissue proteins. In addition, for immunohistological analysis of interest for this thesis, the type of chemical reaction between formalin and protein enhances nuclear staining opposed to cytoplasmic staining. The tissues were transferred to 70% alcohol to remove formalin and subsequently stored in 70% alcohol until processing. During processing the tissues were put through a series of increasing concentrations of ethyl alcohol to remove water which was within the spaces of the protein network. The tissues were then immersed in xylene which removes ethyl alcohol from the tissues, as this is immiscible with paraffin and then infiltrated with paraffin which provided a supporting matrix for the tissue. The resulting blocks were cut into either 5 or 6μm sections using a microtome. Creases were removed from the sections by floating out onto a water bath (40°C) which also aided the transfer of sections to Superfrost plus™ microscope slides (BDH Laboratory Supplies, Dorset, UK). Sections were allowed to air dry and incubated at 37°C overnight in an incubator. Sections were treated with xylene (2 x 5mins) to remove the wax and rehydrated through decreasing concentrations of alcohol (100, 95, 70%, 2mins each) to water to rehydrate the sections.

2.6.2 Histological analysis

2.6.2.1 Van Geison Stain

As a result of coronary artery ligation (section 2.3.1.3), cardiomyocytes become terminally damaged and are replaced histologically by collagen and fiborous scar tissue. The deposition of scar tissue formed in the infracted zone of the left ventricle from CAL mice was confirmed using van Geison's stain for collagen.

Briefly, 5μm sections of hearts were immersed in celestin blue for 5mins, rinsed in tap water and subsequently stained in Mayer's Haemalum for 5mins before being rinsed again in water. Staining was differentiated by a brief immersion in 1% hydrochloric acid/ 70% alcohol mix and then rinsed in water. Thereafter, sections were immersed in van Geison for 3mins, rinsed very briefly in water and dehydrated in absolute alcohol (2 x 5mins) before being transferred to xylene (2 x 5mins). Finally, the slides were dried and coverslips were mounted with DPX mounting medium.

2.6.3 Immunohistochemistry

Sections were pressure cooked for 20mins in citrate buffer (0.1M, pH6), washed in water and then treated with 3% H_2O_2 at room temperature for 30mins to dampen any endogenous peroxidase activity in the tissue. The tissue was washed twice in Tris-buffered saline for 5mins and then treated to block endogenous biotin activity by incubating with blocking serum (rabbit serum (1 part)/Tris-buffered saline (TBS) (4 part)/5% BSA) containing avidin at room temperature for 30mins. Sections were washed and incubated under the same conditions for 15mins with TBS containing biotin. Following washing with TBS, sections were incubated overnight at 4°C, with primary antibody, *Table 2.2*, all antibodies were diluted in blocking serum. On the second day, the first of every two TBS washes was done using 0.05% Tween/TBS. The addition of the Tween detergent helps to eliminate background. The primary antibody was washed off before the sections were incubated for 30mins at room temperature with the biotinylated secondary antibody, *Table 2.2,* and then washed to remove any excess unbound secondary antibody. Sections were incubated for 30mins with ABC-HRP, which binds to the biotinylated secondary antibody. Sections were then washed and bound secondary antibody visualised using 3-3'-diaminobenzidine (DAB) a substrate which reacts with HRP to generate a brown stain and the colour change was monitored microscopically. DAB is active for 5min and can be inactivated in samples in which staining becomes apparent during that time by washing with water. The reaction can be repeated for a second time in samples in which no colour change was observed once the initial application of DAB has been

washed off. If no staining appears following a second exposure, it is unlikely that subsequent exposures to DAB will result in a colour change. The tissues were then counterstained with nuclear stain, Harris' haemotoxylin. The sections were placed in haemotoxylin for 3mins before being washed in water and rinsed in acid/alcohol for 5s to remove any excess haemotoxylin. Tissues were rinsed in water and dehydrated by placing in increasing concentrations of alcohol, (70, 95, 100% up to 100%) and xylene before being mounted using Pertex mounting medium.

Antibody	Blocking Serum	Primary antibody	Secondary antibody
ERα Novacastra (NCL-ER-6F11)	Rabbit	Monoclonal mouse anti- human	Rabbit anti- mouse
ERα DAKO	Rabbit	Monoclonal mouse anti- human	Rabbit anti- mouse
ERß Novacastra (NCL-ER-beta)	Rabbit	Monoclonal mouse anti- human	Rabbit anti- mouse
ERß in- house (Saunders)	Rabbit	Polyclonal sheep anti- human	Rabbit anti- sheep

Table 2.2 ***Antibodies used for the expression of ERα and ER ß in the mouse cardiovascular system***

The primary and secondary antibodies used for the detection of ERs in the mouse cardiovascular system and the blocking serum used.

2.7 Calculations and statistical analysis

2.7.1 Calculations

The dose ratio (DR) is the ratio by which the agonist concentration has to be increased in the presence of a second drug to restore a given level of response. Dose ratio (DR= D_1/D_2) was calculated for the contractile response to PE in the presence of indomethacin by dividing the concentration of PE that produced a given contractile response (D_1) by the concentration of PE in the presence of indomethacin that produced the same contractile response (D_2).

2.7.2 Statistical analysis

Statistical analysis was carried out using GraphPad 'Prism' software (CA, USA). Statistical significance was calculated by two-way ANOVA followed by a Bonferroni post hoc test. Unpaired t-test was also used where appropriate. Significance level was taken at $P< 0.05$.

2.8 Solutions, drugs and chemicals.

Salts were purchased from Sigma- Aldrich Company, Ltd., Dorset, UK, unless otherwise stated.

Solutions for molecular biology

Tail Buffer

1.0M Tris- HCl adjusted to pH 8, 0.5M EDTA adjusted to pH 8, 1.0M NaCl, 10% SDS

TE Buffer

10μM Tris, 1μM EDTA, adjusted to pH 8 with concentrated hydrochloric acid

DEPC H2O

5 drops of diethyl pyrocarbonate (DEPC) were added to 500mls of distilled H2O. The bottle was then shaken vigorously for 30 seconds and left to stand for 20 minutes. The solution was then shaken again and left for 1 hour before being autoclaved.

TBE

TBE contained 0.45M Tris base, 0.445M Boric Acid and 0.5M EDTA (pH 8.0) made up to 1l with sterile water.

Buffers

Saline
0.9w/v sodium chloride solution

Physiological salt solution
118mM NaCl, 4.7mM KCl, 25mM $NaHCO_3$, 1.17mM KH_2PO_4, 1.2mM $MgSO_4$ in distilled water, pH 7.4.

A x10 stock solution was made up and stored at 4°C. On experiment day, the stock was diluted and supplemented with 5.5mM glucose, 0.03mM EDTA and either 1.6mM $CaCl_2$ for the male vascular study, *Chapter 4*, and 2.5mM for the female vascular study, *Chapter 5*.

60mM Potassium Chloride salt solution
62.7NaCl, 60mM KCl, 25mM $NaHCO_3$, 1.17mM KH_2PO_4, 1.2mM $MgSO_4$ in distilled water, pH 7.4.

A x5 stock solution was made up and stored at 4°C. On an experiment day, the stock solution was diluted and supplemented with 5.5mM glucose, 0.03mM EDTA and either 1.6mM $CaCl_2$ for the male vascular study, *Chapter 4,* and 2.5mM for the female vascular study, *Chapter 5*.

Drugs

All stock concentrations of drugs used were made up in deionised water and stored at -20°C unless otherwise stated. On experiment day, drugs were diluted to required concentration using physiological salt solution.

Indomethacin
Stock concentration of drug was dissolved in 4%$NaHCO_3$ and stored until required at -20°C.

Noradrenaline

Stock solution of NAdr (10^{-2}M) also contained ascorbic acid (10^{-1}M). The stock solution was stored at -20°C until required.

Org 44488

Dr Darcey Black (Organon, Newhouse, Scotland) generously supplied Org 44488

Histological stains

Van Geison

100ml saturated aqueous picric acid and 10ml 1% Acid Fuschin

Celestin Blue

2.5g Celestin Blue B, 25g Ferric Ammonium Sulphate, 70ml Glycerol in 500ml distilled H_2O.

Chapter 3

Immunolocalisation of ERs in the mouse cardiovascular system

3.1 Introduction

Oestrogen induces cellular responses in target tissues by binding to specific oestrogen receptors. The first of the two receptors to be cloned, from a human breast cancer cell line was, ER, later designated ERα, (Green *et al*, 1986). Shortly afterwards, ERα cDNA clone was isolated from the mouse uterus which shared 97% and 88% homology with ERα protein sequences isolated from human and rat respectively (White *et al*, 1987). It was believed for many years that this receptor was the sole mediator of oestrogen action on target cells until a second ER was discovered in the rat prostate (Kuiper *et al* 1996) and mouse ovary (Tremblay *et al*, 1997). This second ER was designated ERß to distinguish it from ERα.

Following the identification of ERs, immunohistochemical and molecular biological techniques were employed to determine their distribution and cellular localisation. Initially, this was predominantly in the reproductive system but was also extended to other tissues including those of the cardiovascular system (Orimo *et al*, 1993, Leiberman *et al*, 1990, Karas *et al*, 1994, Iafrati *et al*, 1997) as research in that area increased.

In the vasculature, ERα was shown to be expressed in rat aortic smooth muscle cells (Orimo *et al*, 1993) and also human vascular smooth muscle cells (Bausero *et al*, 2000). However, in contrast to this, ERα was not expressed in either the aorta or carotid artery of the guinea- pig (Leiberman *et al*, 1990). This suggested that there was a degree of species variation in the expression of ERα in the vasculature. In addition to the expression in vascular smooth muscle cells, ERα was also localised to the nuclei of endothelial cells (Diano *et al*, 1999, Critchley *et al*, 2001) and in the heart, localised to nuclei of cardiomyocytes and coronary arteries of both rat and rabbit (Lou *et al*, 1998, Saunders *et al*, 1997, Neudling *et al*, 2001).

Immunohistochemical and RT-PCR analysis of female rat vascular beds demonstrated the expression of ERß protein in the nuclei of vascular smooth muscle and endothelial

cells of the aorta, tail and uterine arteries (Andersson *et al,* 2001, Lindner *et al*, 1998) and ERß mRNA in the rat carotid artery (Makela *et al*, 1999). Similar to ERα, ERß was expressed in the nuclei of cardiomyocytes and coronary arteries of both rat and rabbit (Lou *et al*, 1998, Saunders *et al*, 1997, Neudling *et al*, 2001).

At the time of this study, little work had been published on the expression of ERs in the cardiovascular system of the mouse. Karas *et al* had demonstrated that ERα and ERß were detectable by RT-PCR in mouse aorta (1999). However, as mRNA was extracted from whole tissue this does not provide information as to the cellular localisation of these receptors. As the literature suggests that expression of ERs in the cardiovascular system may be species dependent, we felt it necessary firstly, to determine the tissue distribution and cellular localisation of ERs in the mouse cardiovascular system.

Secondly, as we were using the ßERKO mouse to determine the effects of oestrogen on the cardiovascular system mediated through ERß, we wished to confirm that these animals did not express immunoreactive ERß.

Oestrogen has been shown to be protective against the development of atherosclerosis (Adams *et al*, 2002, Potier *et a,l* 2003) and myocardial infarction (Smith *et al*, 2000) Mice that do not express apolipoprotein E (Apo E-/-), which mediates the binding of lipid particles to lipoprotein receptors for the utilisation and redistribution of lipids, (Pitas *et al*, 1987), suffer from premature atherosclerosis as a result of a severe lipid disorder (Osada *et al*, 2000). Several studies have reported that oestrogen has an atheroprotective effect in Apo E-/- mice such that it dramatically inhibited lesion initiation, and progression (Bourassa *et al*, 1996, Elhage *et al*, 1997, Tse *et al*, 1998). The atheroprotective effect observed in these studies was not associated with a reduction in plasma cholesterol, thus suggesting that the protective effect of oestrogen was not due to an effect on plasma lipids but rather by a direct effect on the vessel wall. Furthermore, literature suggests that the atheroprotective effects of oestrogen are receptor mediated and can in part be to ERα, as lesion size following chronic oestrogen supplementation of

female mice, is significantly greater in mice lacking ERα compared to WT littermates (Hodgin *et al*, 2001).

In mice, coronary artery ligation leads to myocardial infarction and a reduction in left ventricular systolic function (Booth *et al*, 2002) and provides an animal model for studying MI. In this model, chronic oestrogen supplementation was reported to reduce infarct size and cardiomyocyte apoptosis in mouse models of MI (van Eickels *et al*, 2003). The cadioprotective effect of oestrogen to reduce infarct size was shown to ER mediated as the observed reduction in myocardial injury was abrogated by ICI 182, 780, a non-specific ER antagonist (Booth *et al*, 2003).

Although literature suggests that the protective effects of oestrogen in the aforementioned mouse models of atherosclerosis and myocardial infarction was ER mediated, ER expression in either the vasculature or myocardium has not been reported. We hypothesis that ERs are expressed in the vasculature and cardiomyocytes of Apo E-/- and CAL mice respectively. The third aim of this study was a preliminary study to determine whether the technique of immunohistochemisty for the detection of ERβ in the mouse cardiovascular system could be applied for the detection of ERβ in the mouse pathophysiological models of atherosclerosis and myocardial infarction. A second arm to this part of the study included a preliminary investigation as to whether chronic oestrogen supplementation improved cardiac function following MI.

3.2 Methods

3.2.1 Animals

Animals used for this study were as outlined in *Section 2.1*. Briefly, to determine the distribution and cellular localisation of oestrogen receptors (ERs) in the mouse cardiovascular system, wild type (WT, 14- 18wks) male and female littermates of the ßERKO mouse, of a mixed genetic background C57Bl6/ SvJ129, were used.

Immunohistochemical analysis of ER expression in the cardiovascular system of the ßERKO (14- 18wks) was characterised in both male and female mice also.

Immunoexpression of ERs in animal models of pathophysiological conditions were studied. C57Bl6 female mice purchased from Harlan, underwent bilateral ovariectomy as outlined in *Section 2.3.1.1.1* (13- 15 wks) or were allowed to continue cycling, one week prior to CAL surgery to induce myocardial infarction or sham, as outlined in *Section 2.3.1.3.* Cardiac function was assessed by echocardiography as outlined in *Section 2.3.2.2.* Tissues were harvested from animals two weeks post- surgery (16- 18wks).

Apolipoprotein E knock- out female mice (Apo E-/-, 32- 36wks) which are an established model of atherosclerosis (*reviewed in,* Smith and Breslow, 1997, Van Dijk *et al*, 2000, Fazio and Linton, 2001), were kindly made available to us by Dr I Megson who has a colony established at Edinburgh University.

3.2.2 Tissue collection and processing

Animals were euthanased and tissue obtained and processed for immunohistochemistry as outlined in *Section 2.4.1* and *2.6.1.*

Tissues used for immunohistochemical analysis of the mouse cardiovascular system were heart, aorta and mesenteric artery. The positive controls for ERα expression were mouse ovary and mouse uterus (Horigome *et al*, 1988, Korach *et al*, 1988, Hiroi *et al*, 1999) and mouse ovary for ERß (Krege *et al*, 1998, Dupont *et al*, 2000). Positive controls were included in each run. In addition to the positive control for ERß, female WT sections of heart and aorta which had previously stained positive for ERß were included in the runs for the detection of ERß in i) hearts from mice which had undergone coronary artery ligation and ii) aorta from Apo E-/- mice.

3.2.3. Histological analysis

3.2.3.1. Haemotoxylin and Eosin

Ovaries from both WT and ßERKO female mice were stained with haemotoxylin and eosin as outlined in *Section 2.2.2.2*. Dr Norah Spears (University of Edinburgh) kindly analysed the ovarian phenotype.

3.2.3.2. Van Geison

Myocardial infarction was induced in female mice as outlined in *Section 2.3.1.3* by Dr Isam Sharif. The deposition of collagen rich scar tissue formed in the infarcted zone of the left ventricle from CAL mice was confirmed using van Geison's stain for collagen deposition, as outlined in *Section 2.6.2.*

3.2.3.3 Oil Red

Dr Saibal Biswas performed the histology for the detection of aortic fatty streak lesions in female Apo E-/- mice (40wks) using oil red O. Briefly, thoracic aortas were isolated, stripped of adipose tissue and immediately stained in 0.75% Oil red O (Sudan IV) in isopropanol for 15- 20mins. Excess stain was removed in 70% ethanol. Aortas were opened longitudinally and the surface of the lumen examined for visible plaque areas.

3.2.4. Immunohistochemical analysis

3.2.4.1 Immunoexpression of ERα

For the detection of ERα in the mouse CVS, sections of heart, aorta and mesenteric artery from WT, C57Bl6/ SvJ129 female mice were treated as outlined in *Section 2.6.3.* Two different mouse anti-human ERα antibodies, as outlined in *Table 2.2* of *Section 2.6.3*, were used over a range of dilutions, 1:100- 1:20diln. Negative controls, sections which had not been incubated with primary antibody, were also included for each tissue type in each run.

3.2.4.2 Immunoexpression of ERß

Immunoexpression of ERß in the mouse CVS was studied using two anti-ERß antibodies which were directed against different regions of the ERß protein, namely the hinge region and the c-terminus, as outlined in *Table 2.2* of *Section 2.6.3.* Antigen retrieval and treatment for endogenous peroxidase activity in sections from the tissues studied was performed as outlined *Section 2.6.3.* The antibody directed against the hinge region of ERß was used at 1:1000- 1:750 dilution and the antibody directed against the c- terminus was used at 1:100. Negative controls were sections which had not been incubated with primary antibody and were included for each tissue type in each run.

3.3 Results

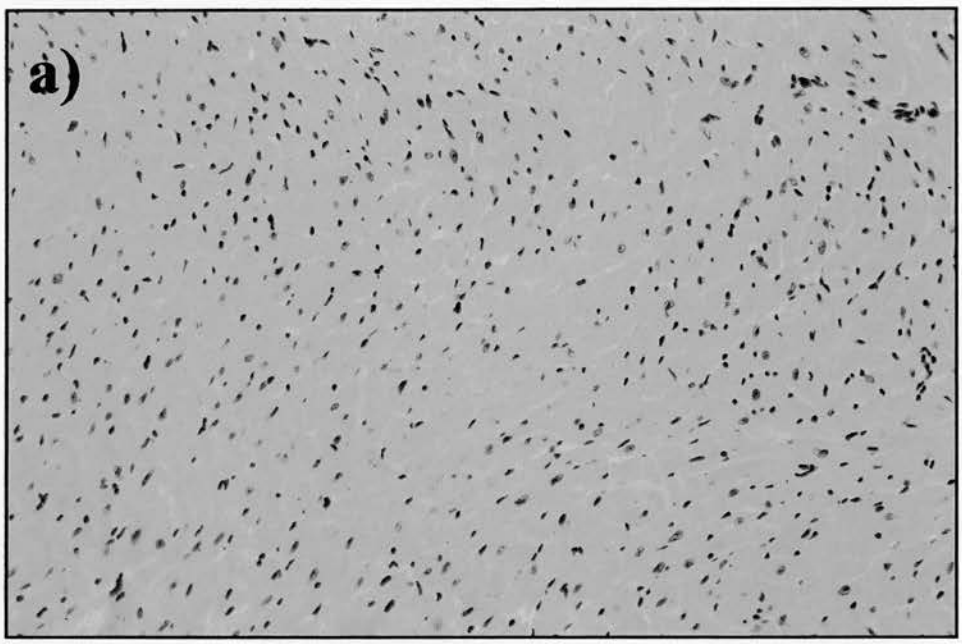
3.3.1 Immunolocalisation of oestrogen receptors in the mouse cardiovascular system

3.3.1.1 Localisation and cellular distribution of ERα

Immunoreactive ERα protein was not detected in the cardiovascular system of female WT mice, using either of the anti ERα antibodies tested, *Figure 3.1*.

Figure 3.1 ***Immunoexpression of ERα in the female mouse cardiovascular system.***

Immunohistochemistry for ERα in the cardiovascular system of normally cycling female WT mice (14- 18wks, n=3). Haemotoxylin stained sections of tissues which were not incubated with anti- ERα antibody a) heart c) aorta and e) mesenteric artery. Immunohistochemistry of b) heart d) aorta and f) mesenteric artery with an anti- ERα antibody. All images are x40 magnification.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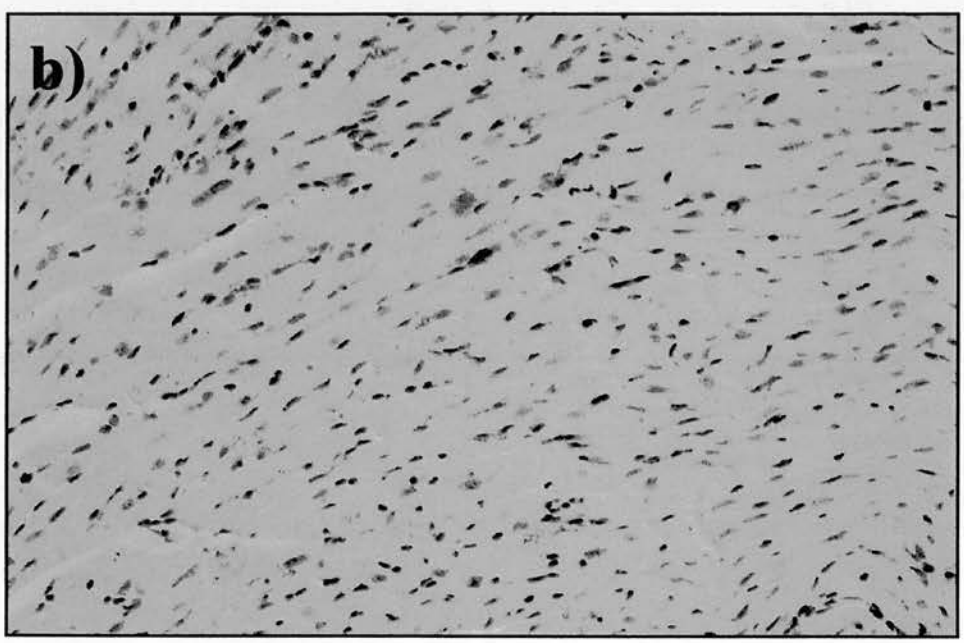
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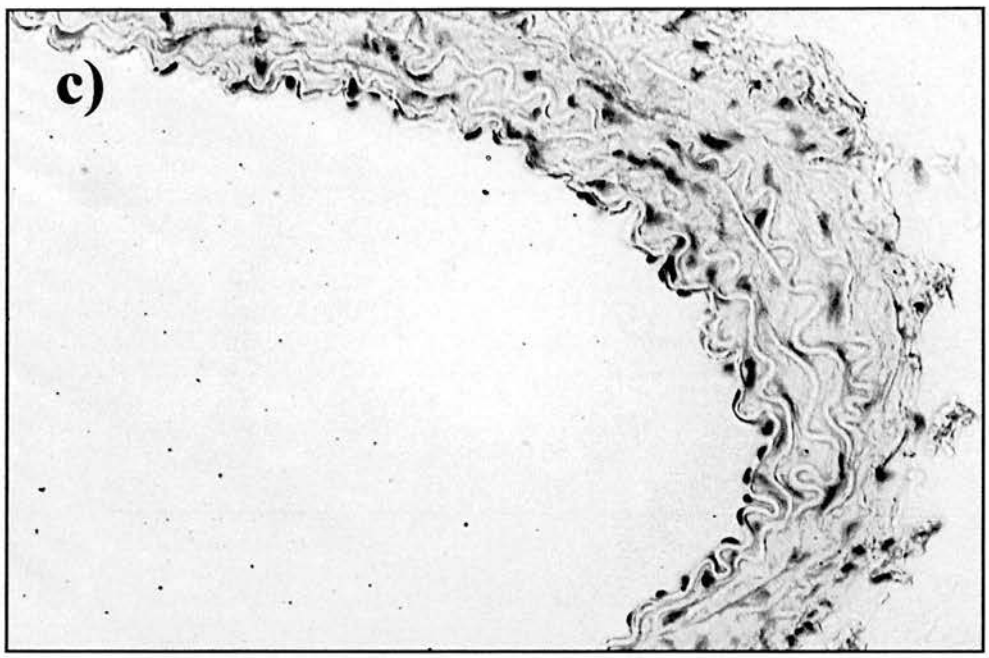
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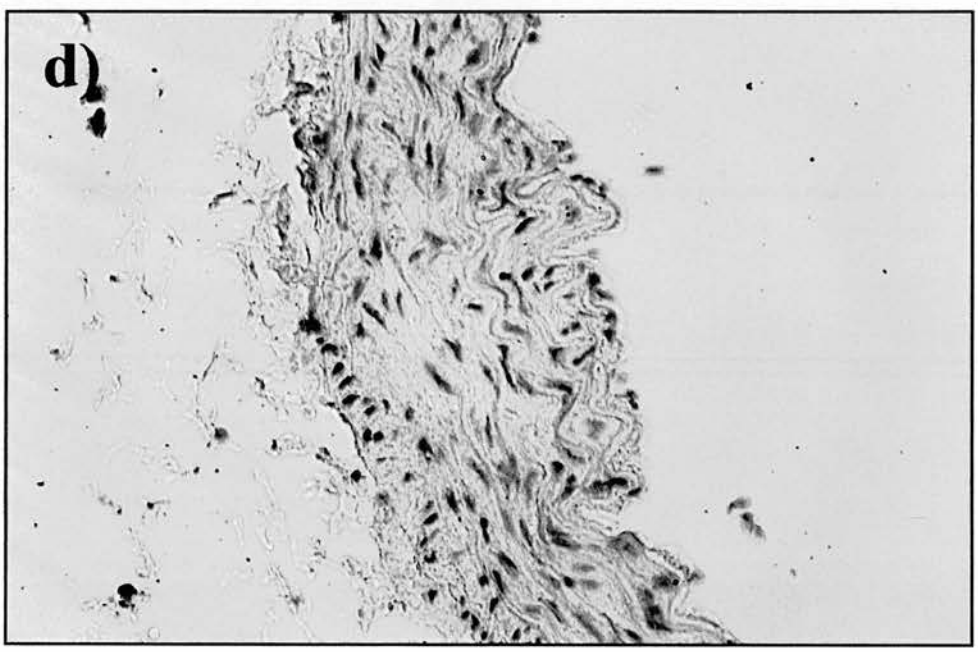
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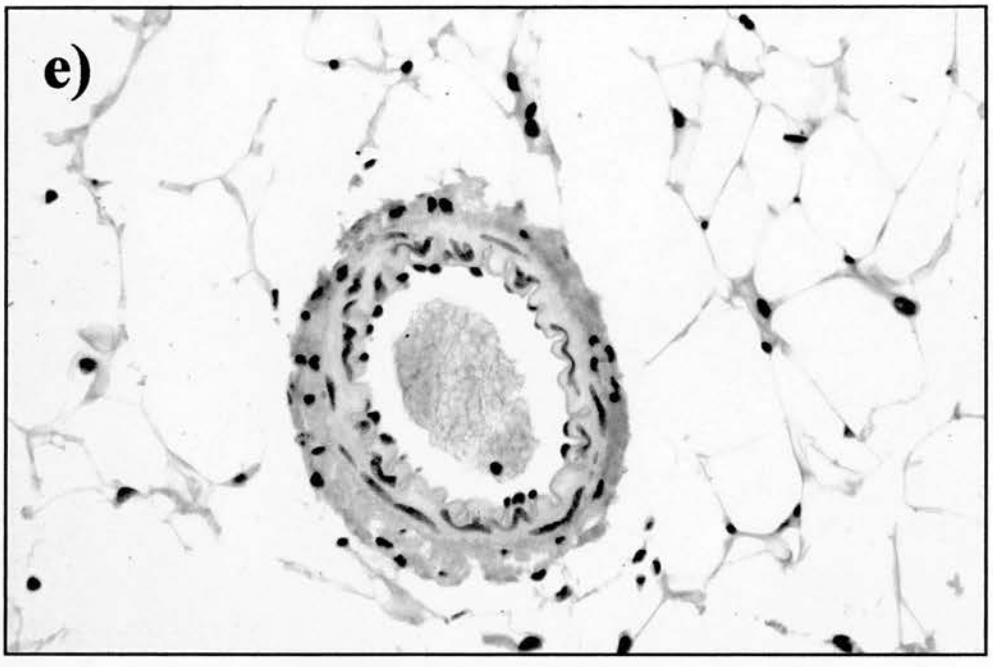
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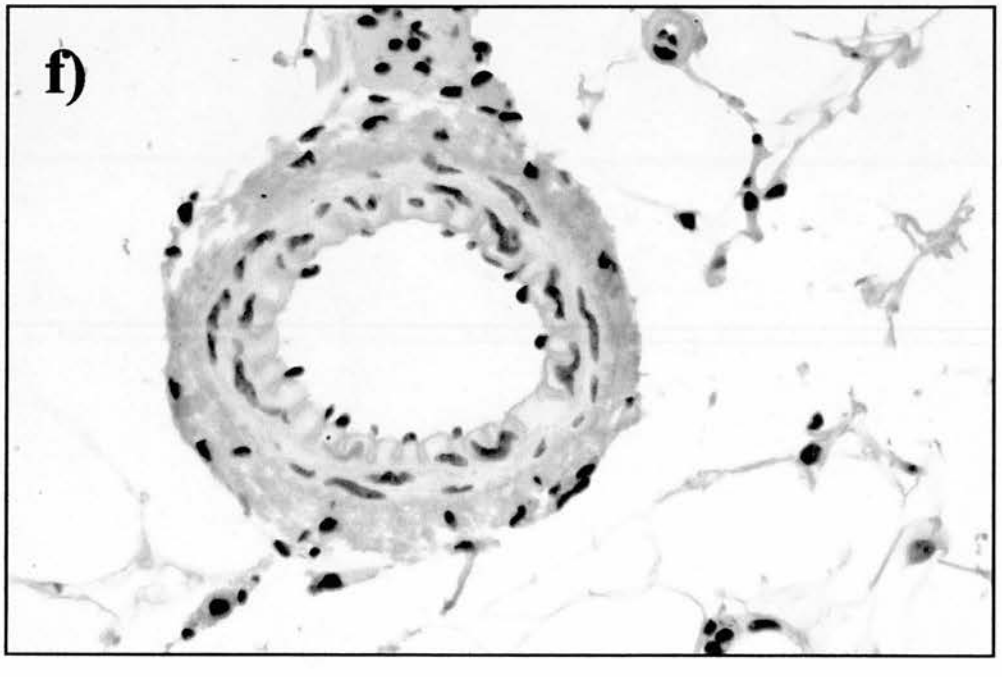
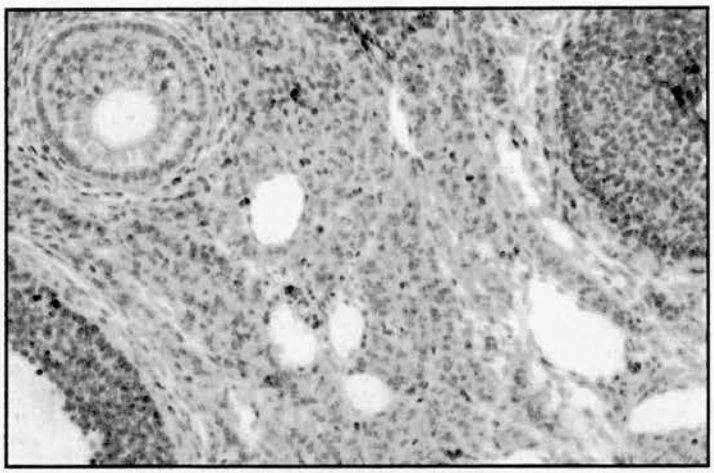

f)

Both antibodies to ERα used in this study were raised against human ERα and therefore to demonstrate that they detected immunoreactive ERα, we tested our conditions in human endometrium. ERα protein was detected in the nuclei of human endometrium using both antibodies, as shown with NCL-ER-6F11 *Figure 3.2*. Secondly, to determine whether both antibodies could cross- react with mouse ERα we repeated the conditions using mouse ovary and found that both antibodies were able to cross- react with mouse ERα. Immunoreactive ERα was detected in the germinal epithelium and ovarian cortex but not found to be expressed in the theca cells, as shown by with NCL-ER-6F11 *Figure 3.2*.

Figure 3.2 ***Immunoexpression of ERα in the mouse ovary and human endometrium***

Immunohistochemistry for ERα in the ovaries of normally cycling female WT mice (14-18wks, n= 3) and human endometrium. a) Haemotoxylin stained section of ovary which was not incubated with anti- ERα antibody, -ve control. Immunohistochemistry of b) mouse ovary and c) human endometrium. Sections a)- c) x20 magnification.

a)



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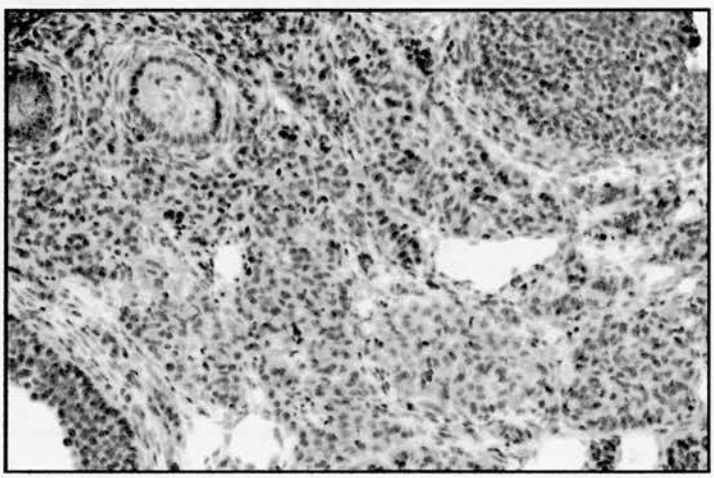

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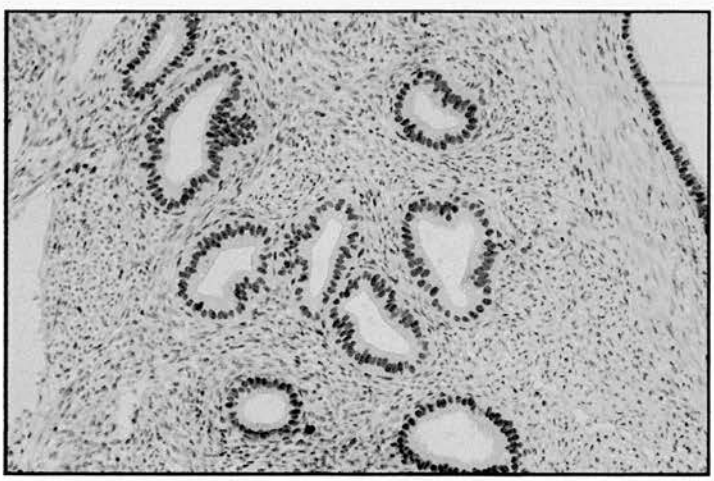


3.3.1.2 Localisation and cellular distribution of ERß

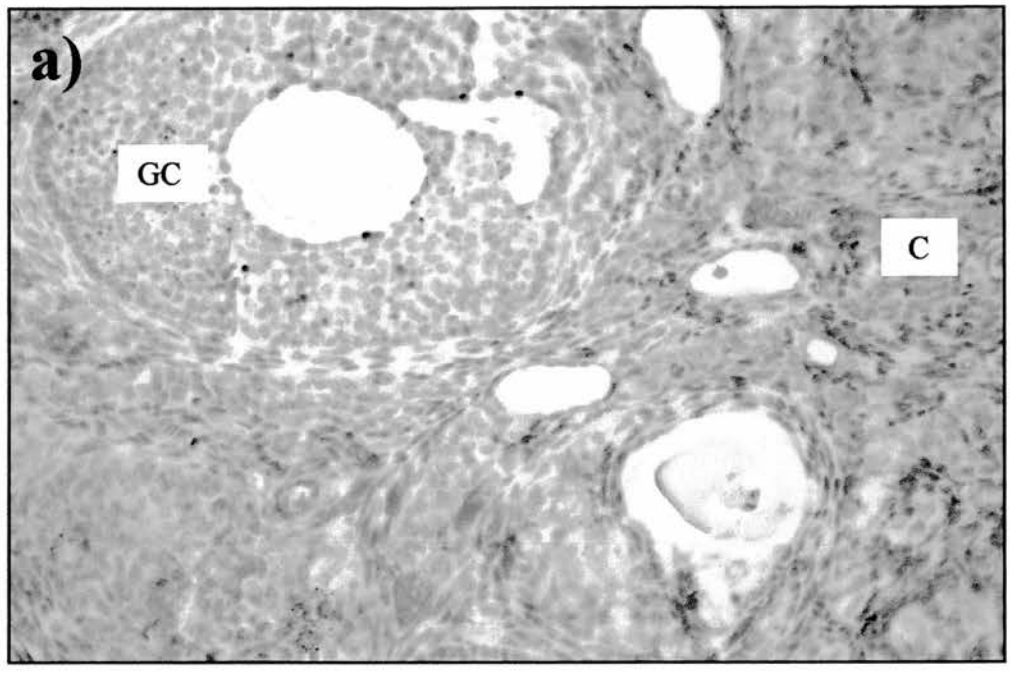
To confirm that the antibody directed against the c-terminus of human ERß was able to detect immunoreactive ERß in mouse tissues, ovaries from female WT mice were immunostained under the conditions used. Immunoreactive ERß protein was detected in the nuclei of granulosa cells of follicles as well as in the ovarian cortex, *Figure 3.3* which is consistent with previously published reports (Krege *et al*, 1998, Schomberg *et al*, 1999, reviewed Saunders *et al*, 1998).

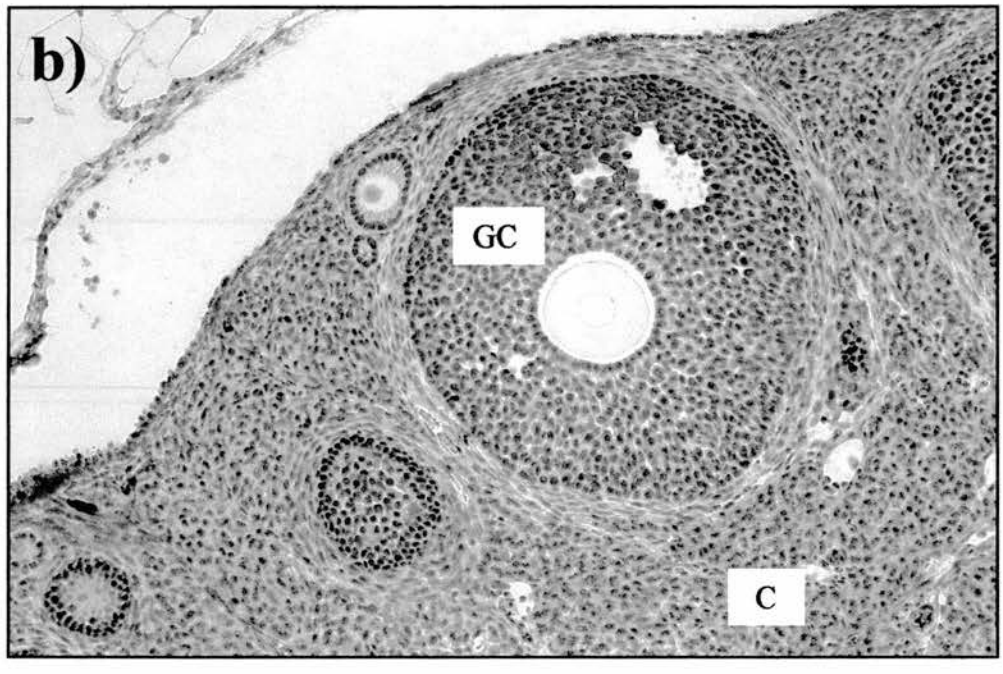
These conditions were then applied for the detection of ERß in the cardiovascular system of both male and female wild-type (C57Bl6/ SvJ129 genetic background) mice.

Immunoreactive ERß was detected in the heart of WT female mice. Expression was localised specifically to the nuclei of cardiomyocytes in the myocardium, *Figure 3.3,* but also to cells of the endocardium and epicardium. Therefore, ERß was expressed diffusely throughout the heart in the majority of cardiomyocytes, although some cardiomyocytes did not stain positive for ERß. Not shown in this figure, ERß was also expressed in coronary arteries, where it was localised to both the endothelium and smooth muscle. No immunoreactive ERß was detected in the cell cytoplasm.

Figure 3.3 ***Immunoexpression of ERβ in the female mouse***

Immunohistochemistry for ERβ in the tissue of normally cycling female WT mice (14-18wks, n= 5). Haemotoxylin stained sections of tissues which were not incubated with anti- ERβ antibody a) ovary and c) heart. Immunocytochemistry of b) ovary and d) heart with the anti- ERβ antibody raised against the c-terminus of the ERβ protein. Images a) and b) are x20 magnification and images c) and d) x40 magnification. (GC) granulosa cells, (C) ovarian cortex.


a)
GC
C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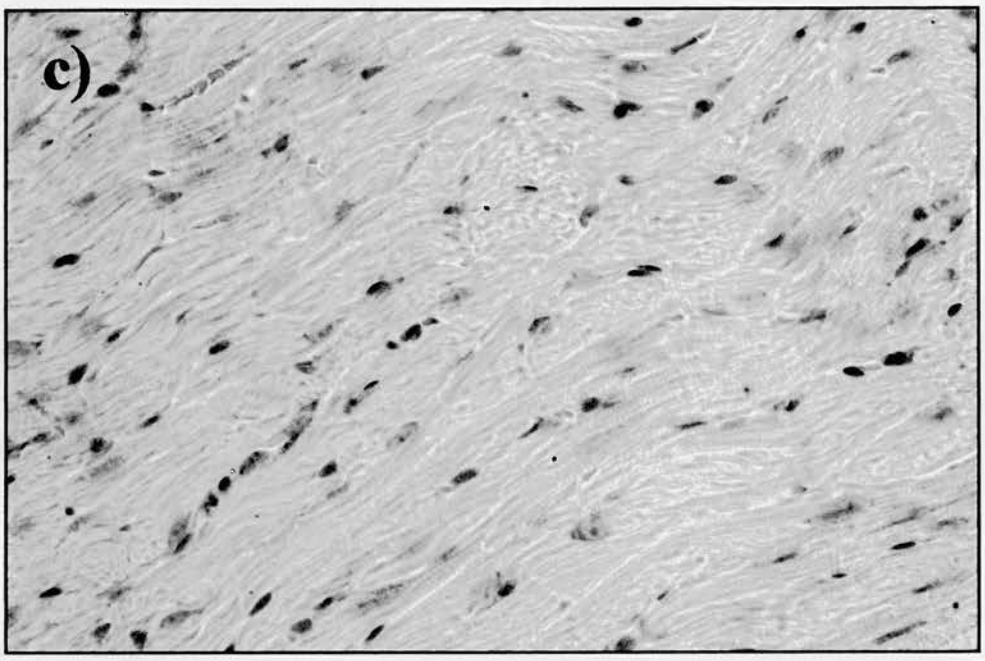
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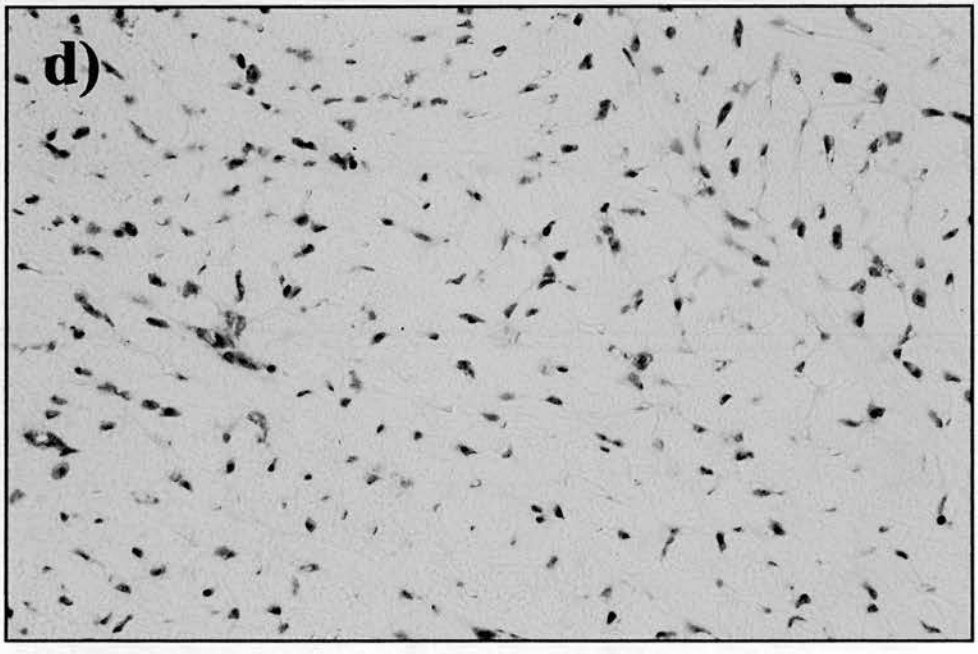
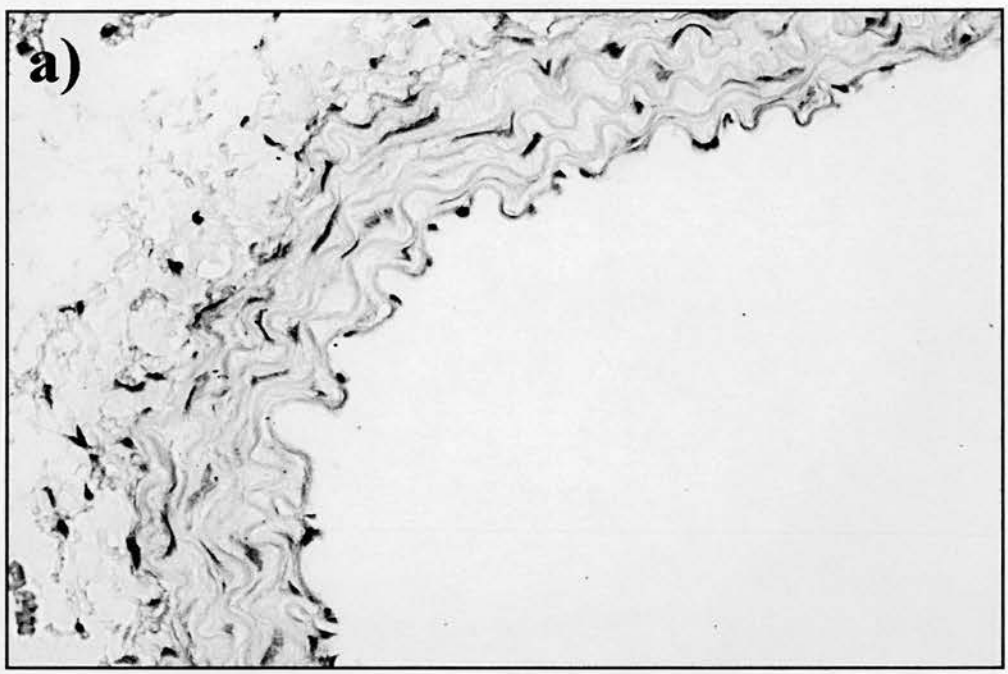

d)

ERß was detected in the aorta, a conduit vessel and the mesenteric artery, a resistance vessel using anti-ERß antibodies directed to the c-terminus of the protein, *Figure 3.4*. The nuclei of endothelial cells, from both vessels studied, expressed immunoreactive ERß. Nuclei of vascular smooth muscle cells also expressed immunoreactive ERß, *Figure 3.4*.

Figure 3.4 ***Immunoexpression of ERβ in the vasculature of the female mouse***

Immunohistochemistry for ERβ in tissue of normally cycling female WT mice (14- 18wks, n= 5). Haemotoxylin stained sections of tissues which were not incubated with anti- ERβ antibody a) aorta and c) mesenteric artery. Immunocytochemistry of b) aorta and d) mesenteric artery with the anti- ERβ antibody directed against the c-terminus of the ERβ protein. Images are all x40 magnification.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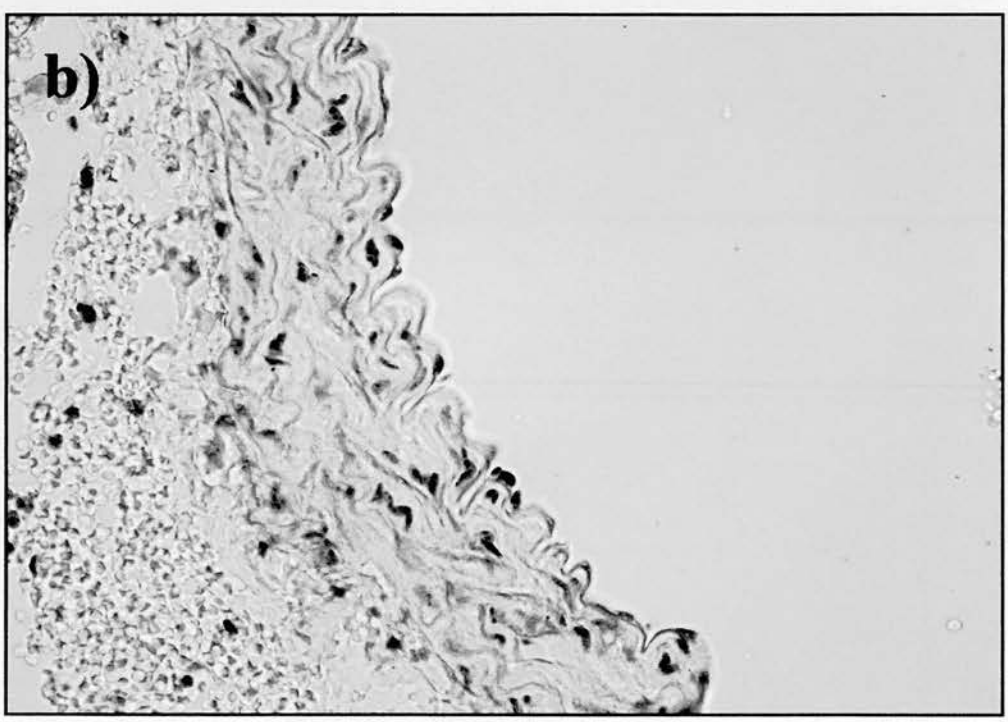
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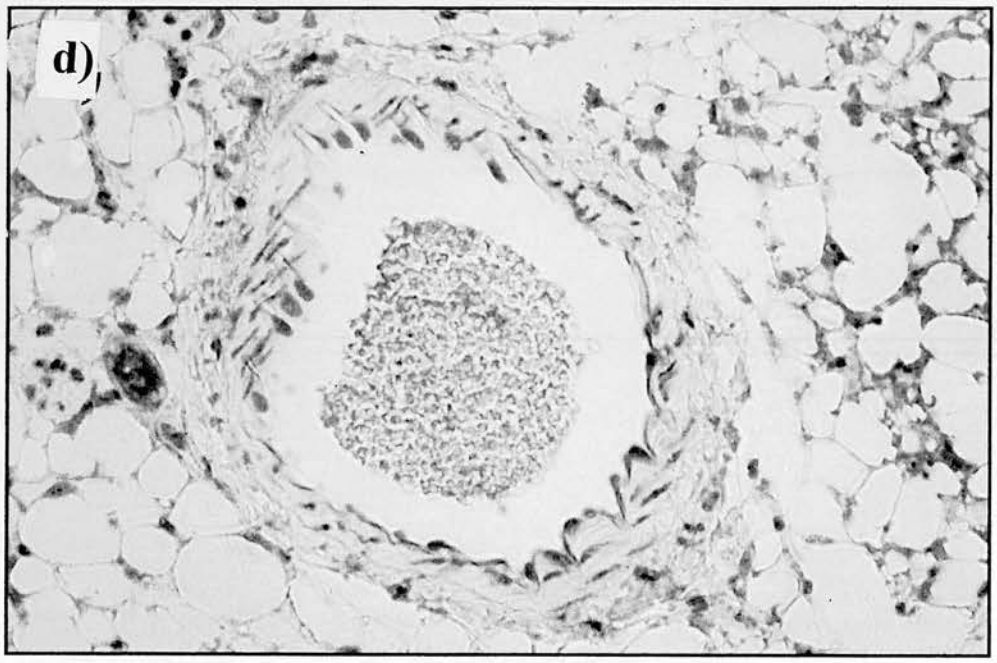

d)

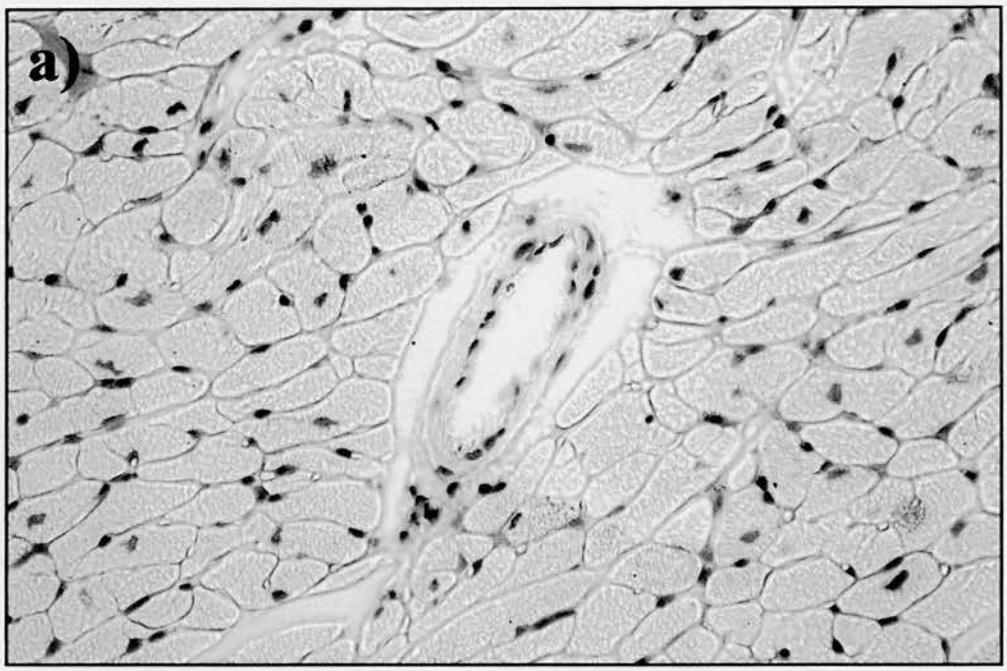
Similar to the expression of ERß in the cardiovascular system of the female mouse, immunoreactive ERß was detected in the nuclei of cardiomyocytes and endothelial and vascular smooth muscle cells of coronary arteries in male mice, *Figure 3.5*.

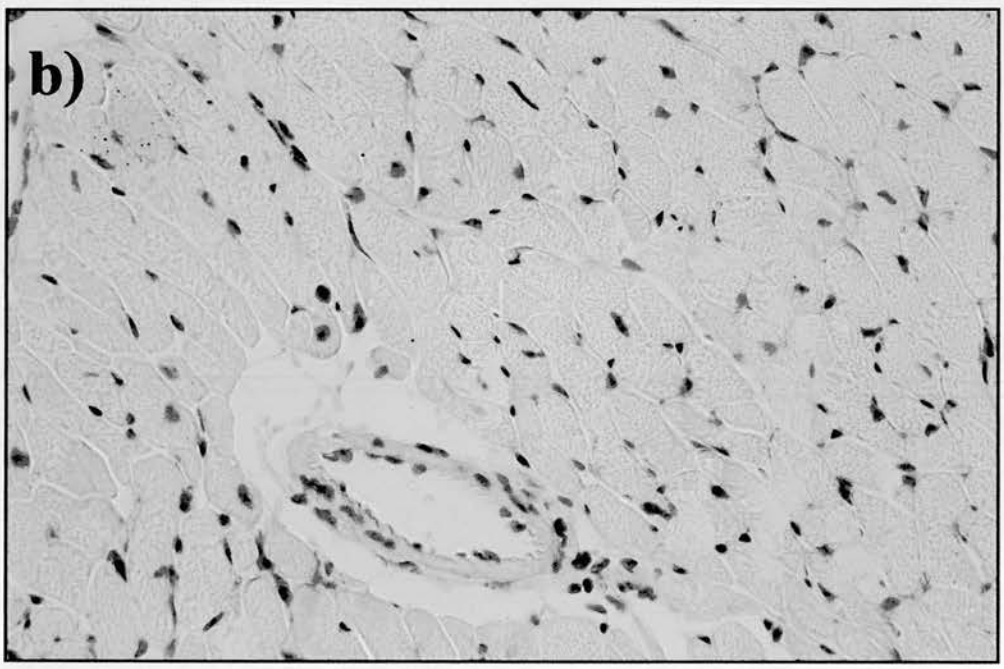
ERß was also detected in endothelial cells and vascular smooth muscle cells of both the aorta and mesenteric artery, *Figure 3.5* which was comparable with that observed in female WT mice.

No immunopositive reaction was detected in negative controls of tissues, where sections had not been incubated with the primary ERß antibody, *Figure 3.4- 3.5*.

Figure 3.5 ***Immunoexpression of ERβ in the male mouse***

Immunohistochemistry for ERβ in tissue of male WT (14- 18wks, n= 5). Haemotoxylin stained sections of tissues which were not incubated with anti- ERβ antibody a) heart, c) aorta and e) mesenteric artery. Immunocytochemistry of b) heart, d) aorta and f) mesenteric artery with an anti- ERβ antibody directed against the c-terminus of the ERβ protein. All images are x40 maginification.


a)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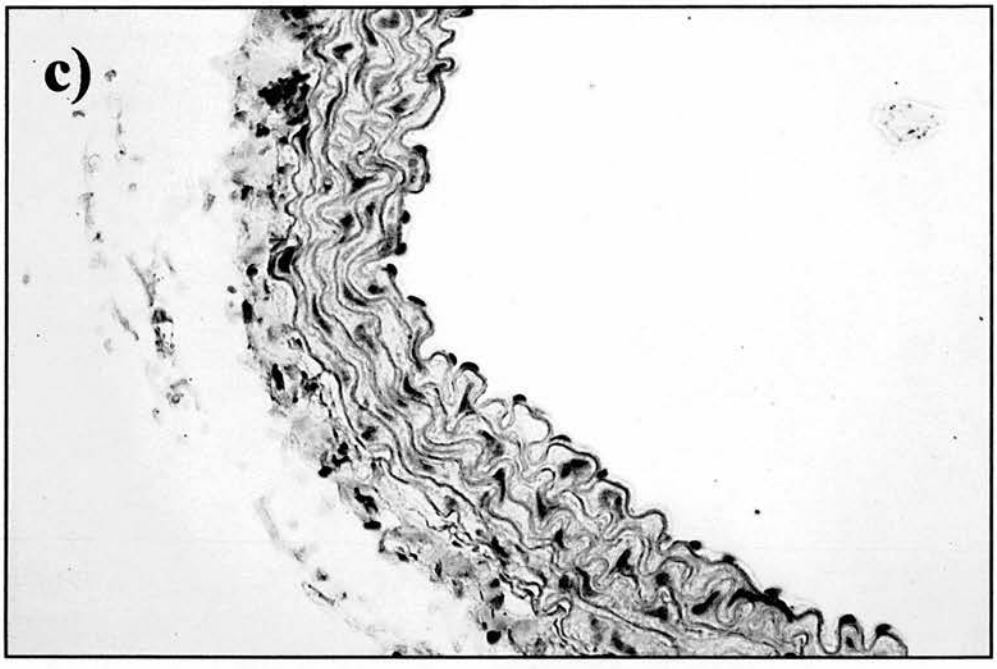
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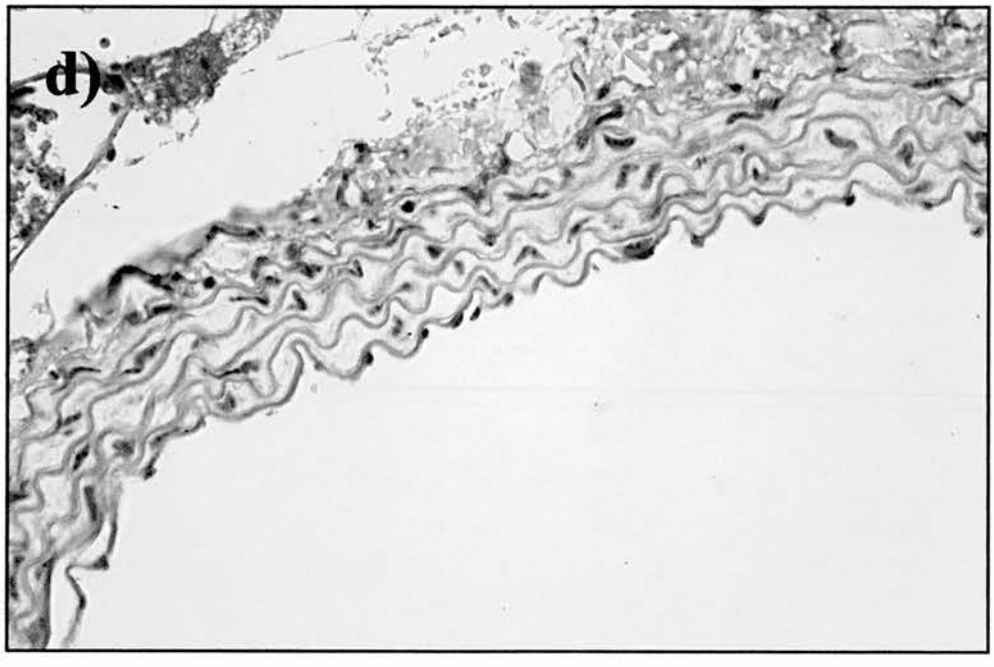
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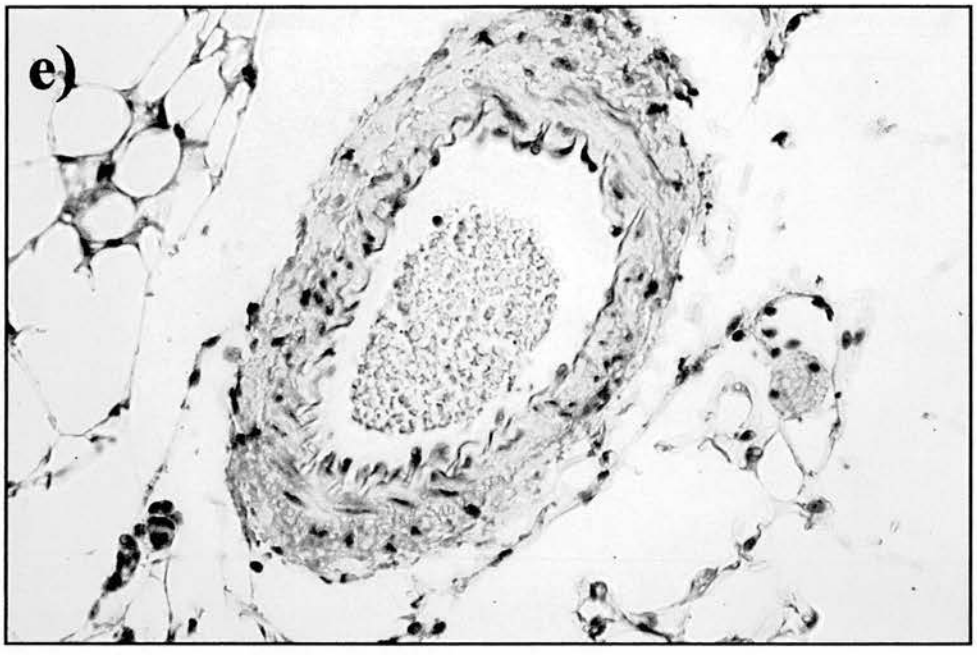
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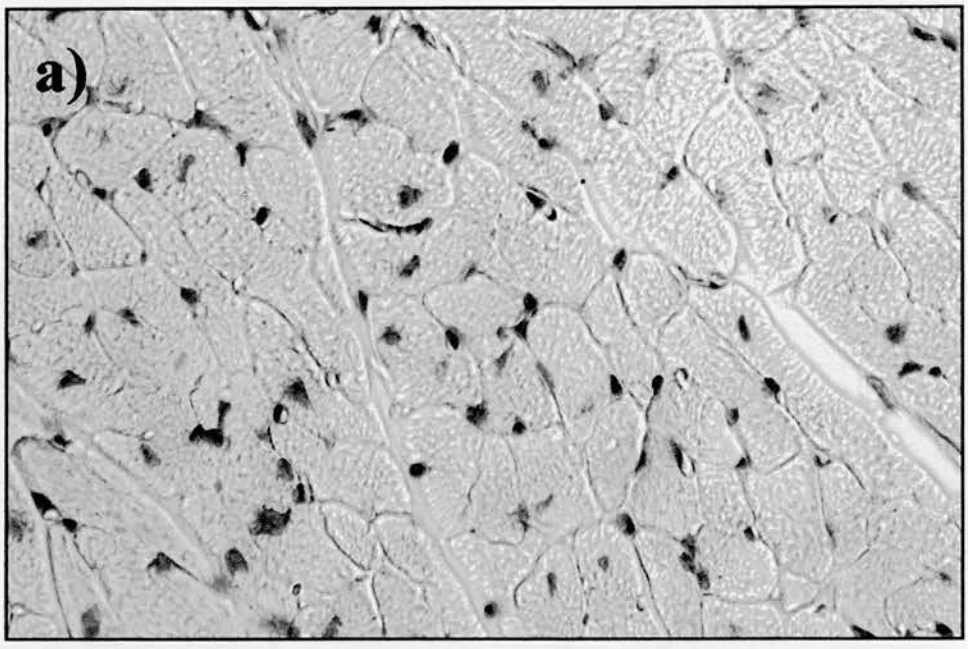
3.3.1.3 Immunoexpression of ERß in the BERKO mouse

Surprisingly, we were able to detect immunoreactive ERß protein in male and female ßERKO mice (Krege *et al*, 1998) using the anti-ERß antibody directed against the c-terminus of the protein, with the same distribution pattern and cellular localisation to that of male and female WT mice. To confirm the expression of ERß protein in the ßERKO mouse we repeated immunohistochemical analysis using a second antibody to ERß, which was directed against a different region of the protein, the hinge region.

Figures 3.6 and 3.7, show that immunoreactive ERß protein was detected in the nuclei of cardiomyocytes, endothelial and vascular smooth muscle cells of the female ßERKO mouse which was independent of the epitope of the antibody used to raise the anti-ERß antibody.

Figure 3.6 ***Immunoexpression of ERβ in the female βERKO mouse***

Immunohistochemistry for ERβ in tissue of normally cycling female βERKO mice (14-18wks, n= 5). a) and c) Haemotoxylin stained sections of heart tissue which were not incubated with the anti-ERβ antibody directed against the hinge region and c-terminus of the ER protein respectively. Immunocytochemistry for the detection of ERβ in section b) antibody directed against the hinge region of ERβ and in section d) using the antibody directed against the c- terminus of the ERβ protein. All images are x40 magnification.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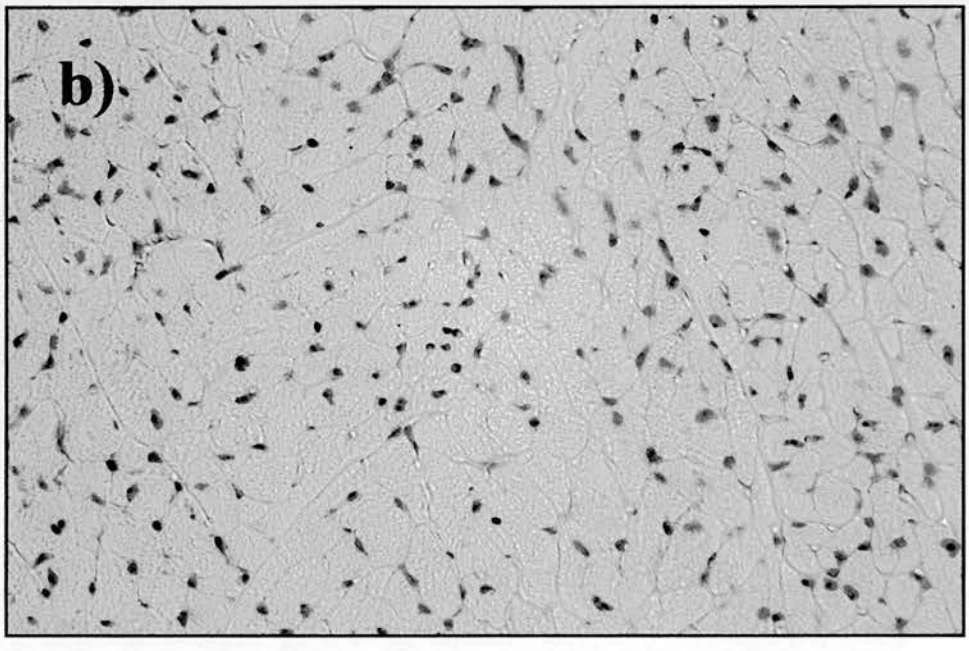
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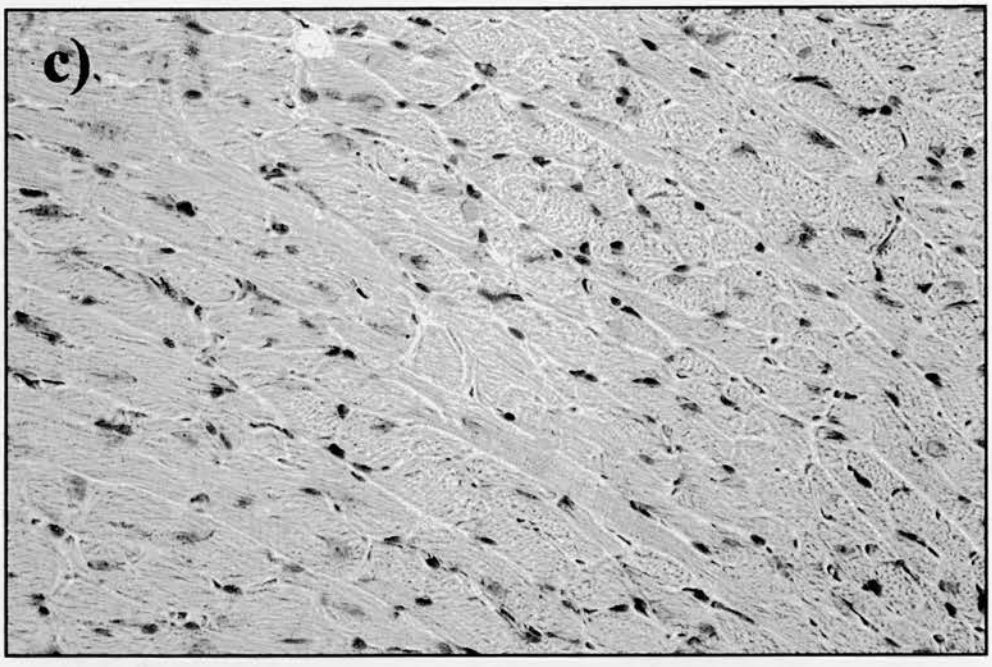
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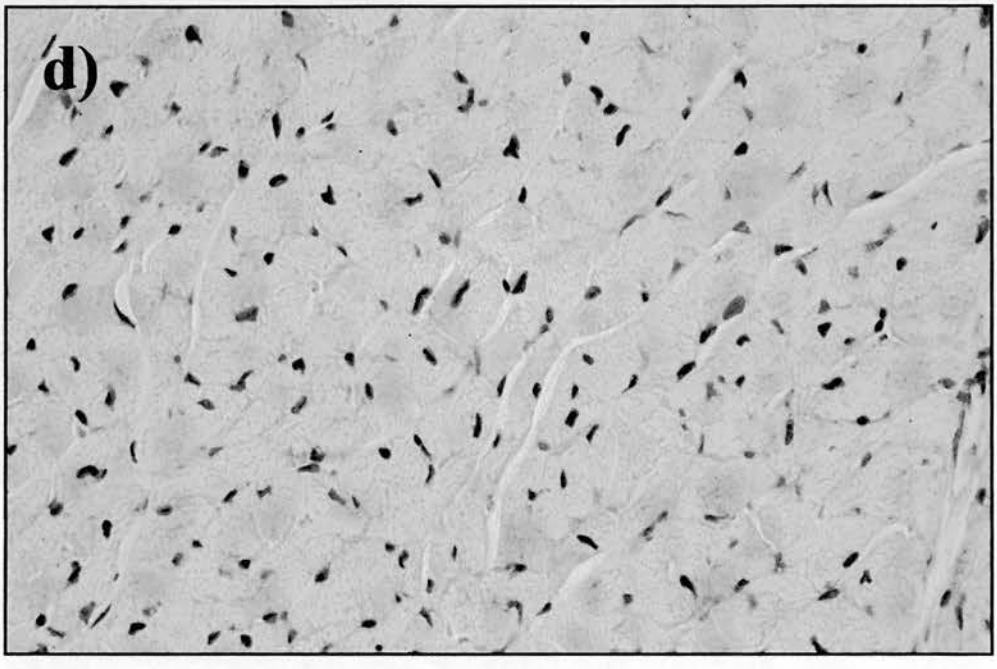
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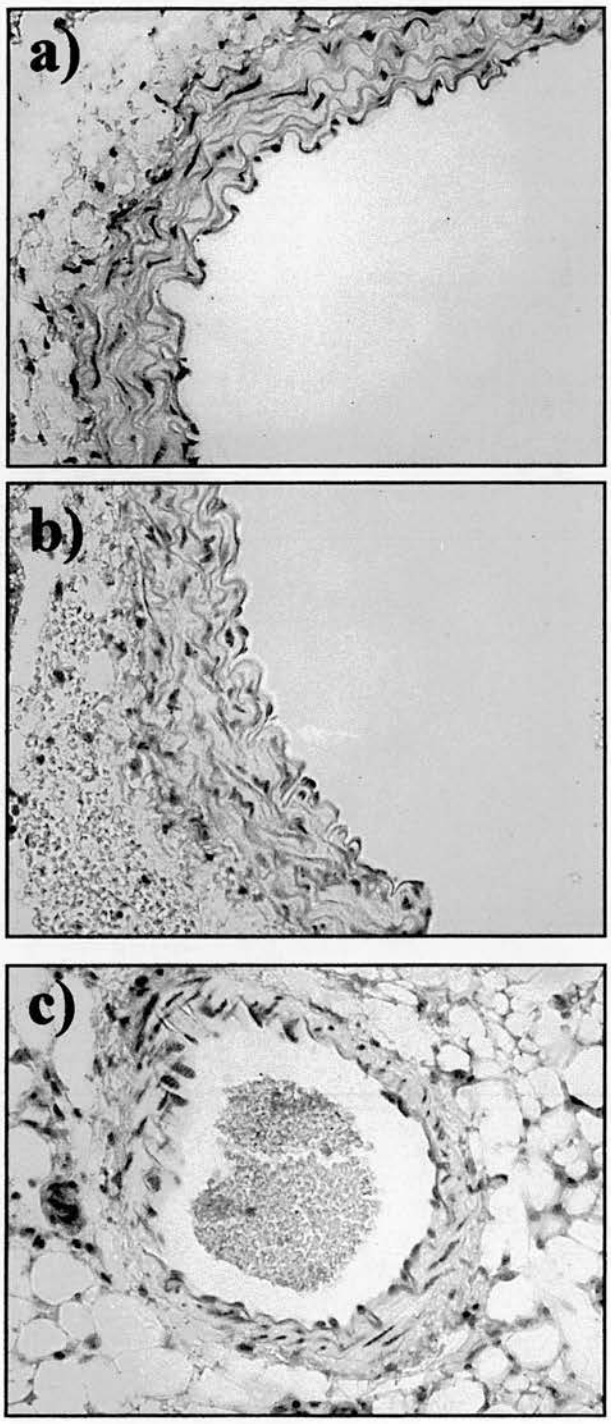
Figure 3.7 ***Immunoexpression of ERß in the vasculature of the female ßERKO mouse***

Immunohistochemistry for ERß in the vasculature of normally cycling female ßERKO mice (14- 18wks, n= 5). a) and d) Haemotoxylin stained sections of aorta which were not incubated with anti- ERß antibody under the conditions used for immunocytochemistry for the antibodies to the c- terminus and hinge region respectively. Immunocytochemistry for the detection of ERß in sections b) and c) used the antibody directed against the c-terminus of ERß and sections e) and f) used the antibody directed against the hinge region of the ERß protein. All images were x40 magnification.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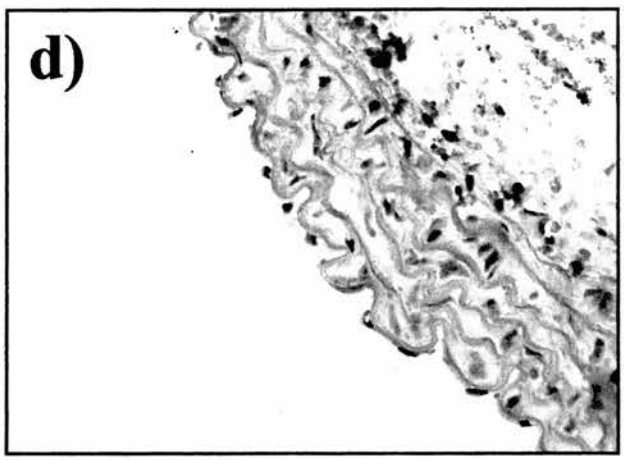
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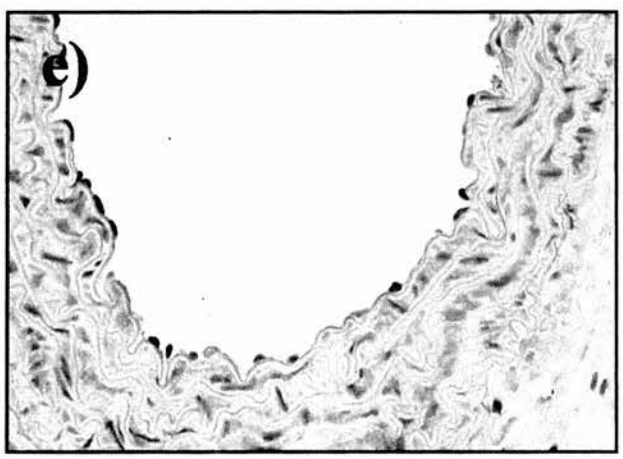
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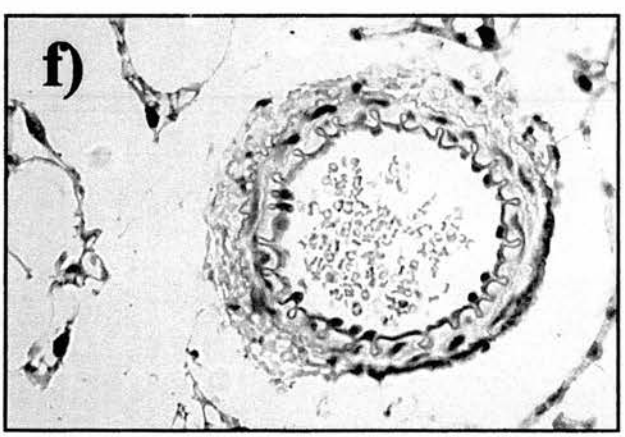
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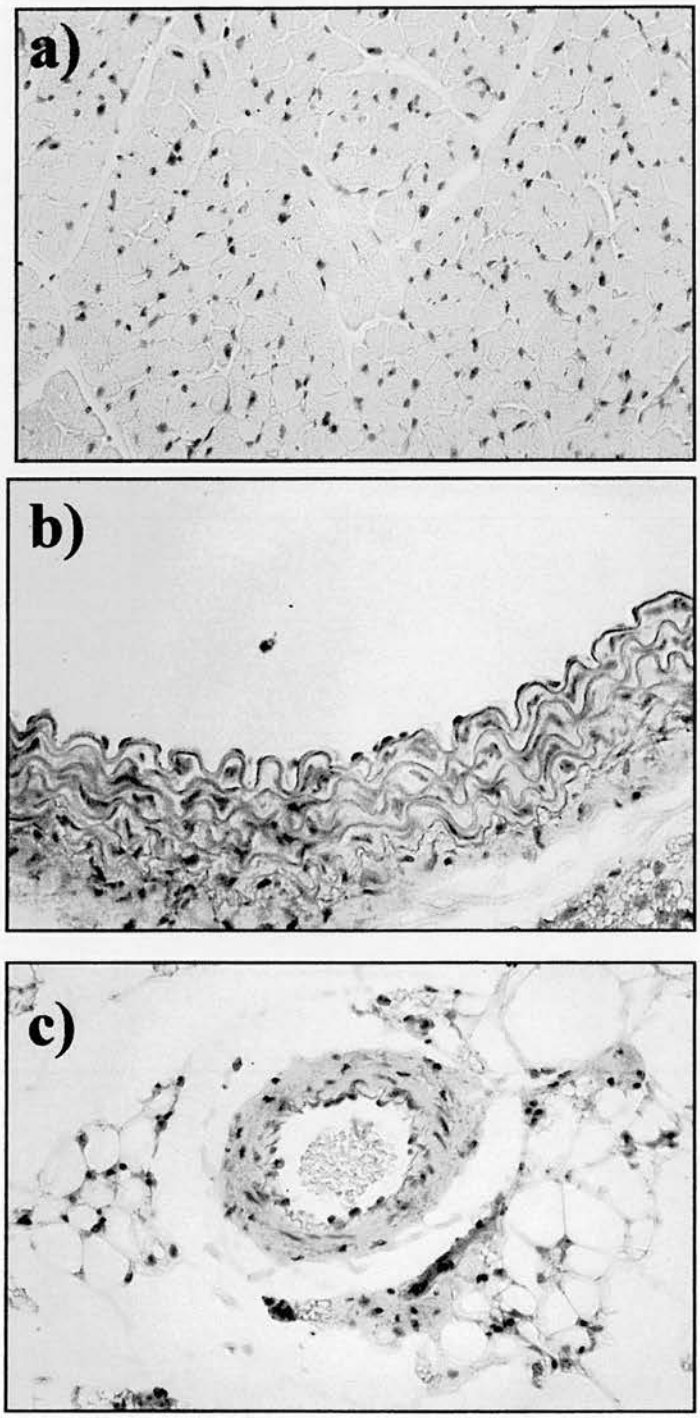
A similar pattern of distribution and cellular localisation was detected in the male ßERKO mouse, *Figure 3.8*.

Figure 3.8 ***Immunoexpression of ERβ in the male βERKO mouse***

Immunohistochemistry for ERβ in the vasculature of male βERKO mice (14-18wks, n= 5). Immunoreactive ERβ protein as detected by the antibody directed against the c-terminus region of ERβ a), b) and c) and the antibody directed against the hinge region d), e) and f). All images are x40 magnification.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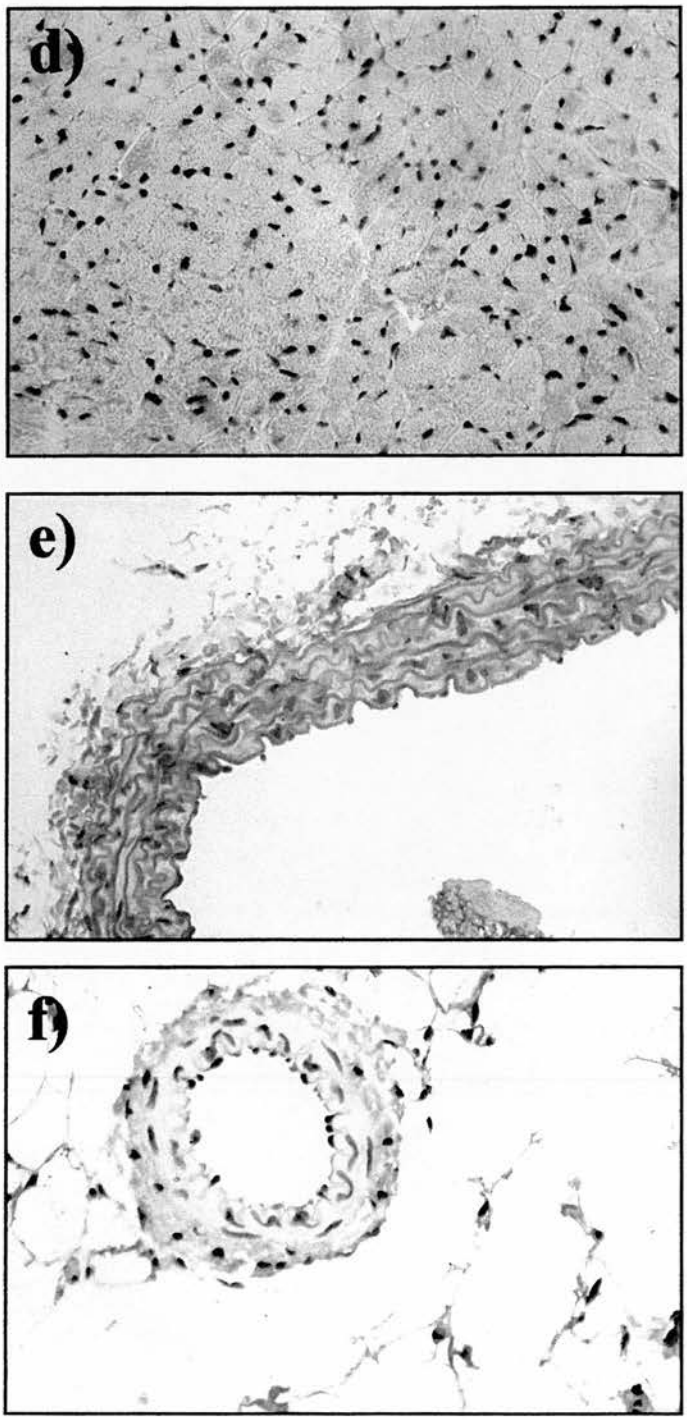
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The βERKO mice generated and described by Krege *et al* were shown not to express immunoreactive ERß protein in their ovaries and to present with an ovarian phenotype (1998). As it was from these mice that our own colony was generated we therefore wanted to confirm whether these mice had the same ovarian phenotype as previously described (Krege *et al*, 1998).

The ßERKO ovaries did exhibit a clear ovarian phenotype when compared to WT littermates, *Figure 3.9a* and *b*. For example the ovaries of ßERKO mice were found to contain more apoptotic cells and to have atresia in the ovarian medulla in comparison to ovaries from WT, *Figure 3.9a* and *b* and SvJ129 (not shown). There was a reduction in the number of corpora lutea in the ßERKO ovaries and those that were present had rough or jagged edges, which indicated that they were degenerating. In addition, the ovaries did not contain any normal corpora lutea which was consistent with a reduction in the number of ovulations. Furthermore, there was an increase in the number of atretic follicles in which the oocyte had either become less spherical in shape or begun to breakdown or pull away from the zona pellusida even before the atrium had developed in the follicle, *Figure 3.9*.

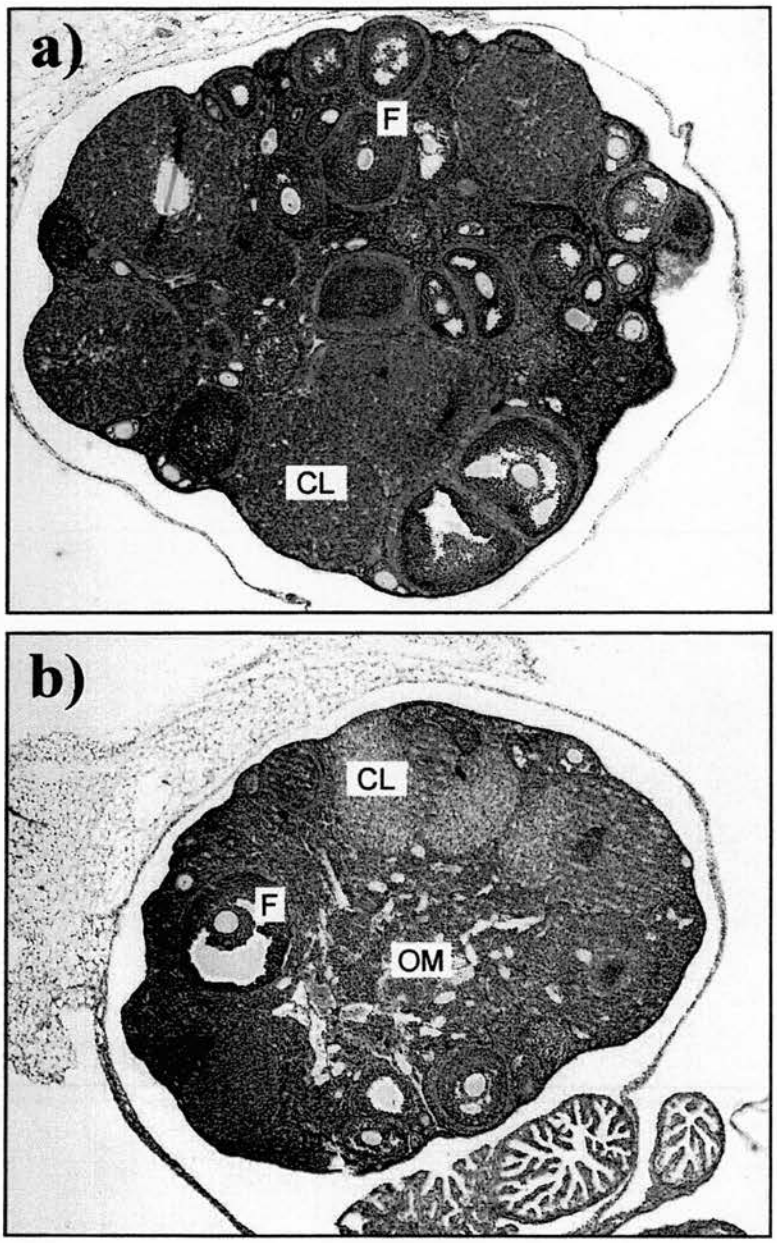


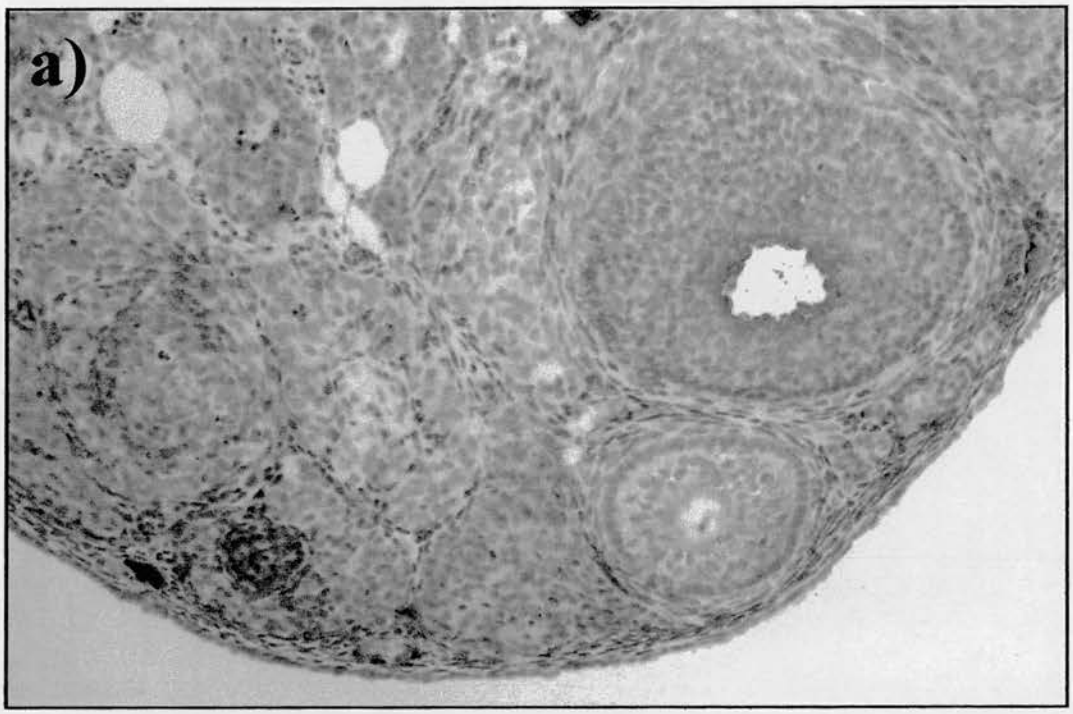
Figure 3.9 ***Morphology of ovaries from female WT and βERKO mice***

Haemotoxylin and eosin stained sections of ovary from normally cycling female mice (14- 18wks, n= 5) a) WT and b) βERKO mice, x5 magnification. (CL) corpus luteum, (OM) ovarian medulla and (F) follicle

Consistent with the results obtained using cardiac tissue, *Figure 3.3*, we also detected immunoreactive ERß in the ovaries from our colony of female ßERKO mice using the antibodies directed against two different regions of the ERß protein, *Figure 3.10*. The cell specific pattern of expression was similar to that of WT mice with similar distribution to that of the WT mouse.

Figure 3.10 ***Immunoexpression of ERβ in the ovary of the female βERKO mouse***

Immunohistochemistry for ERβ in the ovary of normally cycling female βERKO mice (14-18wks, n= 5). Haemotoxylin stained sections of ovary which were not incubated with anti- ERβ antibody formed the negative control for the anti- ERβ antibody directed against a) the c-terminus and c) the hinge region of the ERβ protein respectively. Immunocytochemistry used for the detection of ERβ using an antibody directed against b) the c-terminus and d) the hinge region of the ERβ protein. All images are x20 magnification.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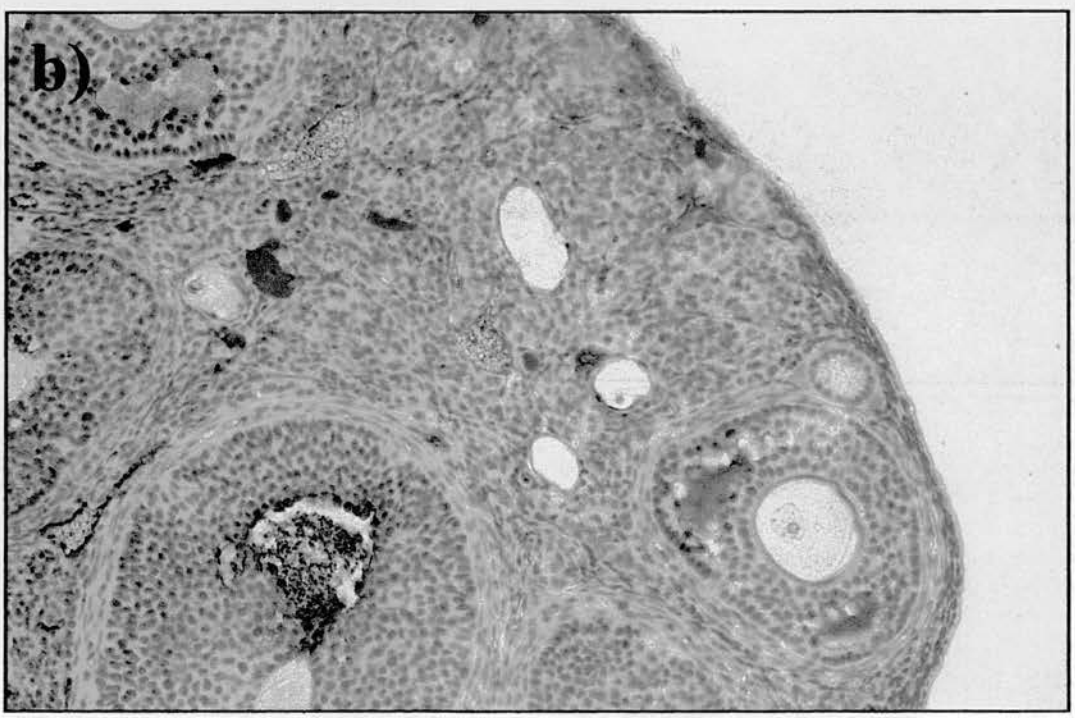
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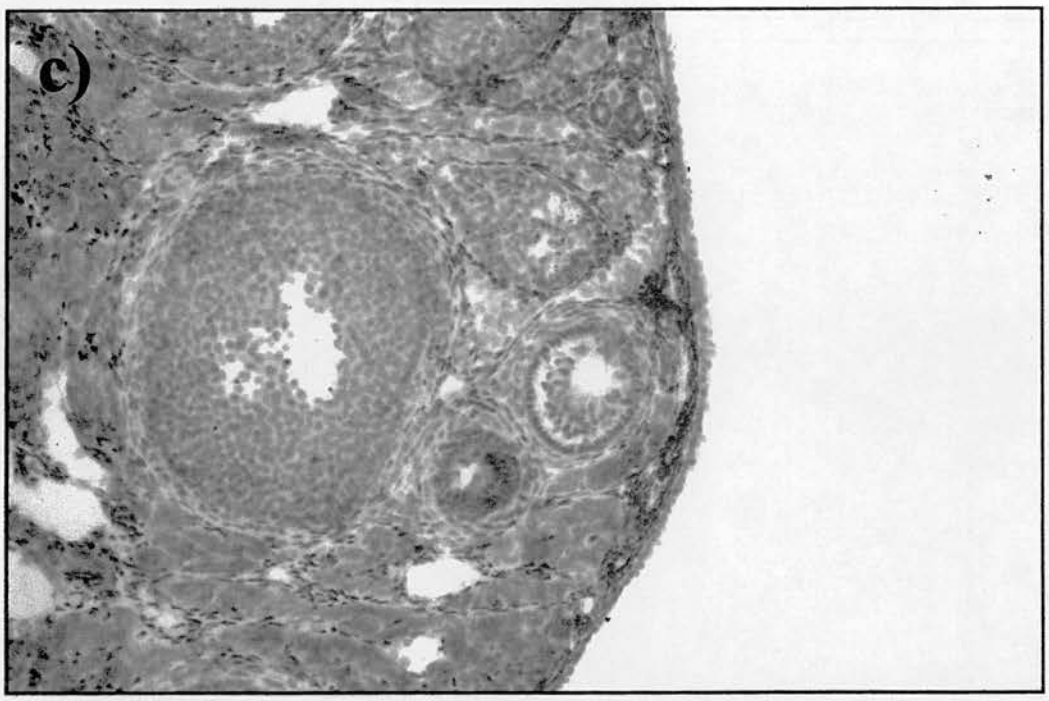
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3.3.1.4 Oestrogen and oestrogen receptors in pathophysiological models.

3.3.1.4.1 Immunoexpression of ERß in a mouse model of atherosclerosis

Specimens containing root, arch and thoracic aorta were stained for neutral lipids and fatty acids using oil red O, *Figure 3.11*. Fatty lesions were deposited in the aortic arch of female Apo E-/- mice aged 40wks.

Aorta from normally cycling female Apo E-/- mice expressed immunoreactive ERß in both endothelial and vascular smooth muscle cells of the aortic arch and thoracic aorta *Figure 3.12*. Immunoreactive ERß was also localised to the nuclei of endothelial cells of the atherosclerotic plaque, *Figure 3.12b*.

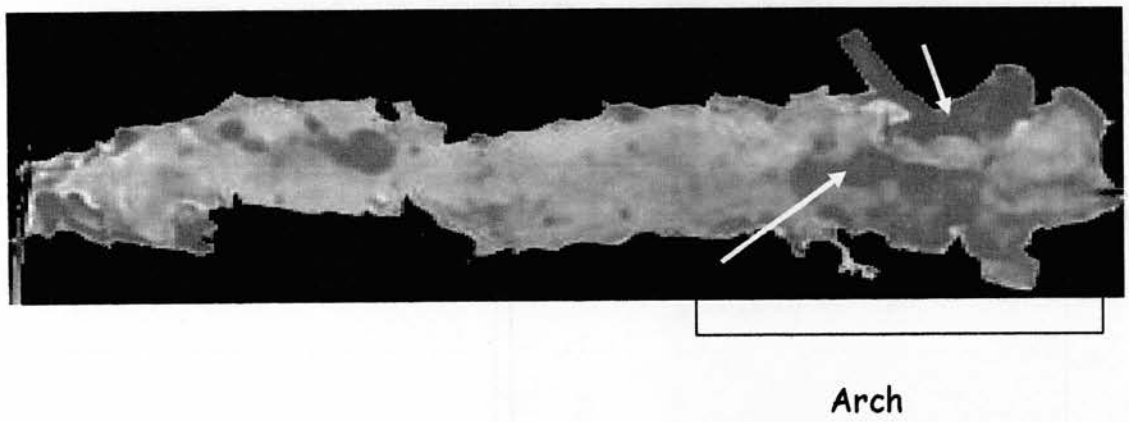
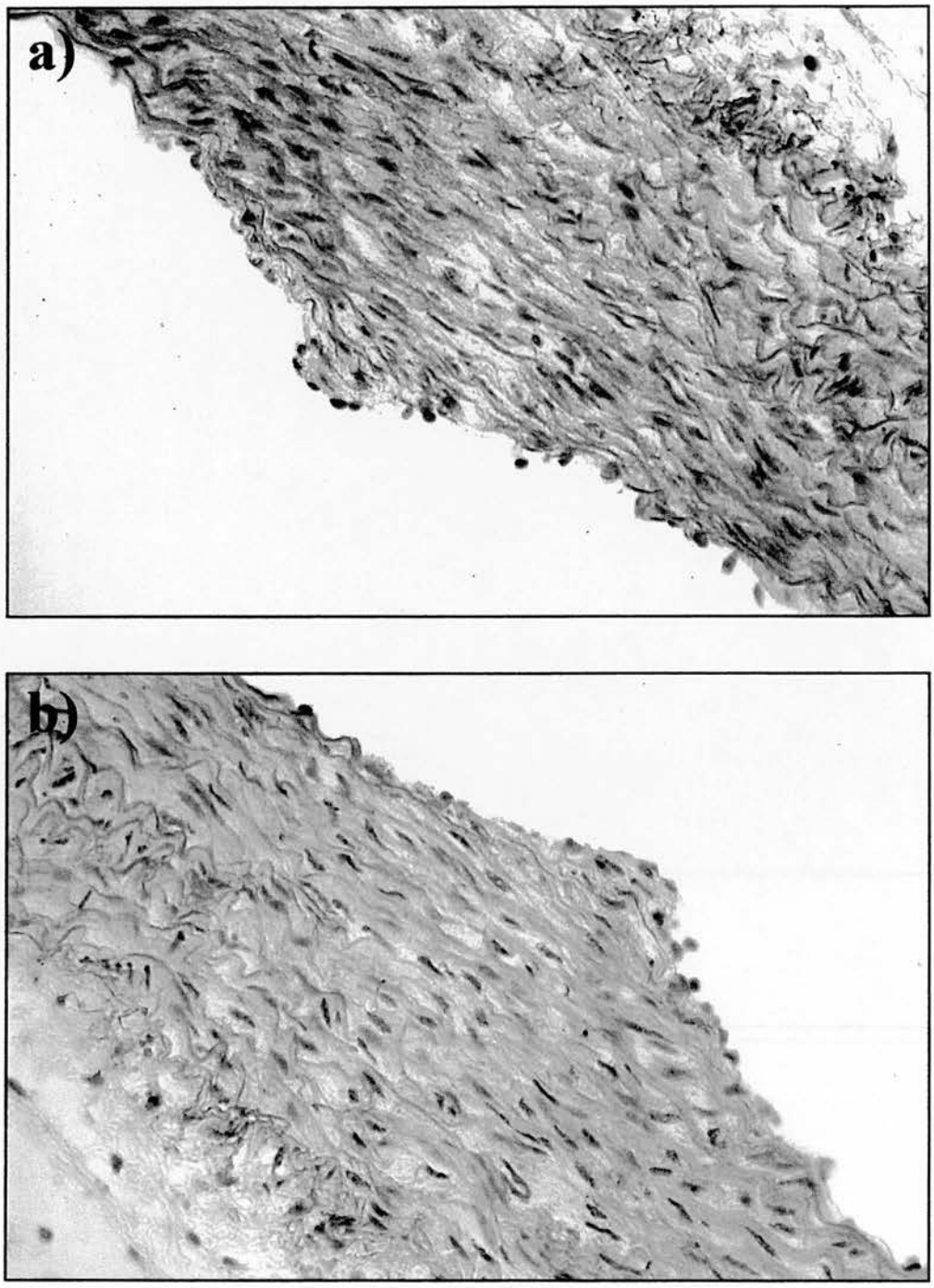


Figure 3.11 ***Detection of fatty lesions in the aorta of an Apo E-/- mouse.***

Aorta from a normally cycling female Apo E-/- (40wks) stained with oil red O. Arrows indicate sites of fatty streak deposition in the aortic arch.

Figure 3.12 ***Immunoexpression of ERß in the aorta of the female Apo E-/- mouse***

Immunohistochemistry for ERß in tissue of normally cycling female Apo E-/- mice (20-22wks, n= 5). Haemotoxylin stained sections of a) aortic arch and c) thoracic aorta which were not incubated with the anti-ERß antibody. Immunocytochemistry for the detection of ERß in sections using an antibody directed against the hinge region of ERß, b) aortic arch, d) thoracic aorta. The aortic arch is the site of the atherosclerotic lesion. All images were x40 magnification.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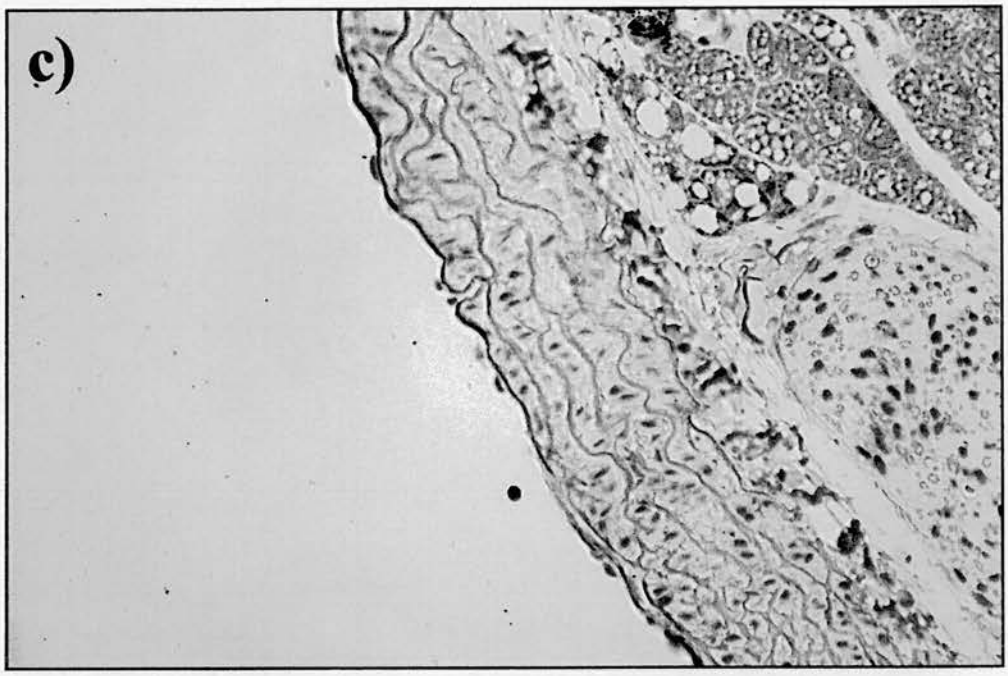
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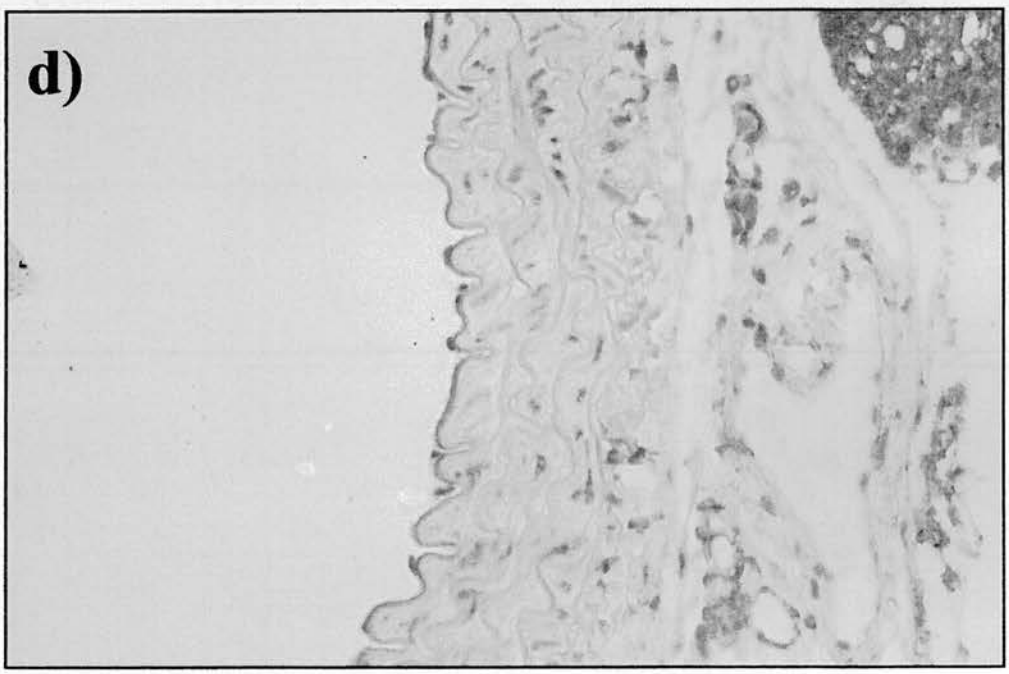
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3.3.1.4.2 Echocardiography and immunodetection of ERß in a mouse model of myocardial infarction

3.3.1.4.2.1 Effect of CAL and oestrogen status on body and tissue weights

Body weight of mice in each of the three oestrogen treatment groups was not significantly different in either CAL or sham operated animals, 2-way ANOVA (surgery x oestrogen status) $p<0.05$, *Table 3.1.*

The uterine weight of female mice which had undergone ovx and received chronic oestrogen supplementation, was significantly greater than that of normally cycling mice and those which had been ovx and administered placebo, *Table 3.1.*

Heart weight had not increased as a result of CAL, despite scar formation, compared to sham operated controls and neither was there a difference in heart weight between animals of different oestrogen status, *Table 3.1.*

3.3.1.4.2.2 Effect of CAL and oestrogen status on cardiac function

In-vivo echocardiography, two weeks post-surgery, demonstrated that CAL led to a reduction in ejection fraction which was not different between female mice of different oestrogen status, *Figure 3.13.*

Fractional shortening was also decreased following CAL and was not different between the three treatment groups. However, the Bonferroni post-hoc test revealed that fractional shortening was significantly reduced following CAL in normally cycling female animals and those which were ovariectomised and supplemented with oestrogen. Although a trend was developing in both the placebo and oestrogen treated animals such that CAL would lead to a reduction in fractional shortening in both of these groups also, *Figure 3.13.*

Weight	Sham			CAL		
	Cycling	Placebo	Oestrogen	Cycling	Placebo	Oestrogen
Body (BW, g)	21.06± 0.41	25.43± 0.64	23.83± 0.94*	21.73± 0.53	25.18± 0.96	25.25± 0.39*
Heart (% BW)	0.60± 0.06	0.64± 0.02	0.54± 0.01	0.67± 0.04	0.68± 0.12	0.64± 0.07
Uterus (%BW)	0.65± 0.07	0.32± 0.06	0.85± 0.15	0.58± 0.04	0.24± 0.04	1.40± 0.13

Table 3.1 ***Body and tissue weights for female mice which had undergone CAL surgery***

*Female WT mice, of different oestrogen status, which had undergone CAL or sham surgery, were weighed at time of being euthanised and tissue harvested was weighed and expressed as a percentage of body weight. Values are expressed as mean± SEM and analysed using a 2- way ANOVA followed by a Bonferroni post- hoc test, *p<0.05 compared to young cohort of animals (Sham cycling, n=5; CAL cycling, n=9; Sham ovx+ placebo, n=3, CAL ovx+ placebo, n=4; Sham ovx+ oestrogen, n= 3 and CAL ovx+ oestrogen, n=6).*

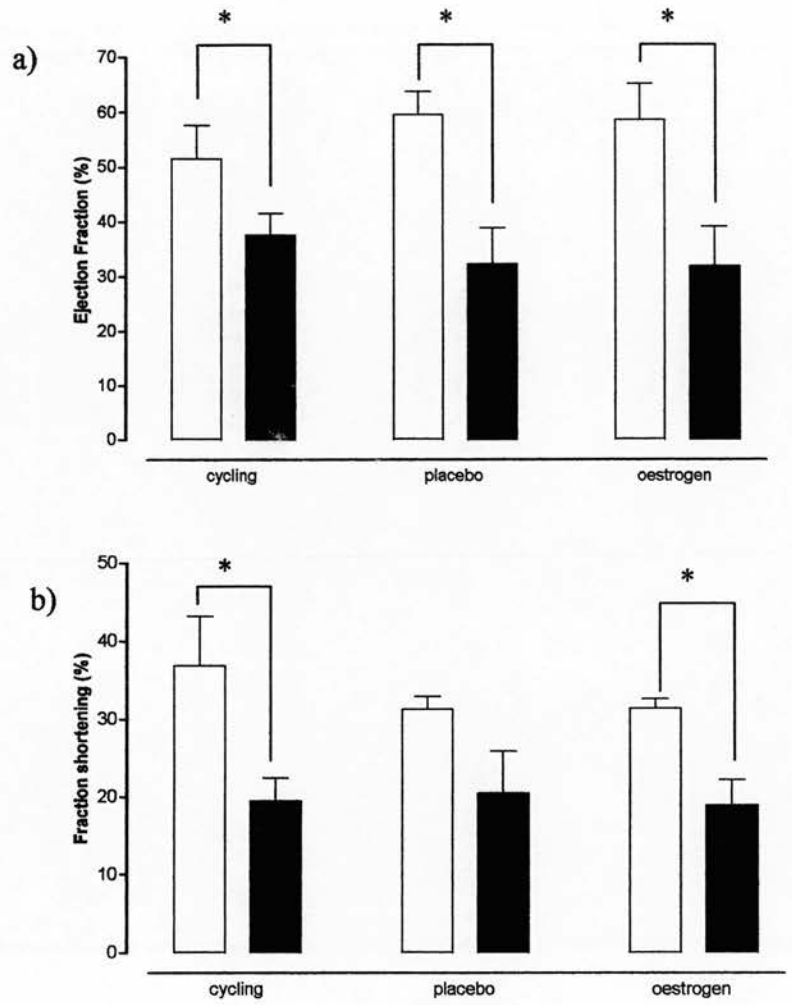


Figure 3.13 ***Assessment of ejection fraction and fractional shortening in female mice which had undergone CAL or sham.***

*Ejection fraction and fractional shortening were assessed in female WT mice (16-18wks) which had undergone either sham surgery, □ or CAL, ■. Female mice, which had undergone surgery, were of different oestrogen treatment; normally cycling or ovx and supplemented with either placebo or oestrogen pellets. Data are expressed as mean± SEM and analysed using a 2-way ANOVA (treatment x surgery) followed by a Bonferroni post- hoc test. *p<0.05 Sham operated animals; cycling n=5, Ovx+ placebo n= 3, Ovx+ oestrogen n= 3, CAL animals; cycling n= 8, Ovx+ placebo n= 4, Ovx+ oestrogen n=6.*

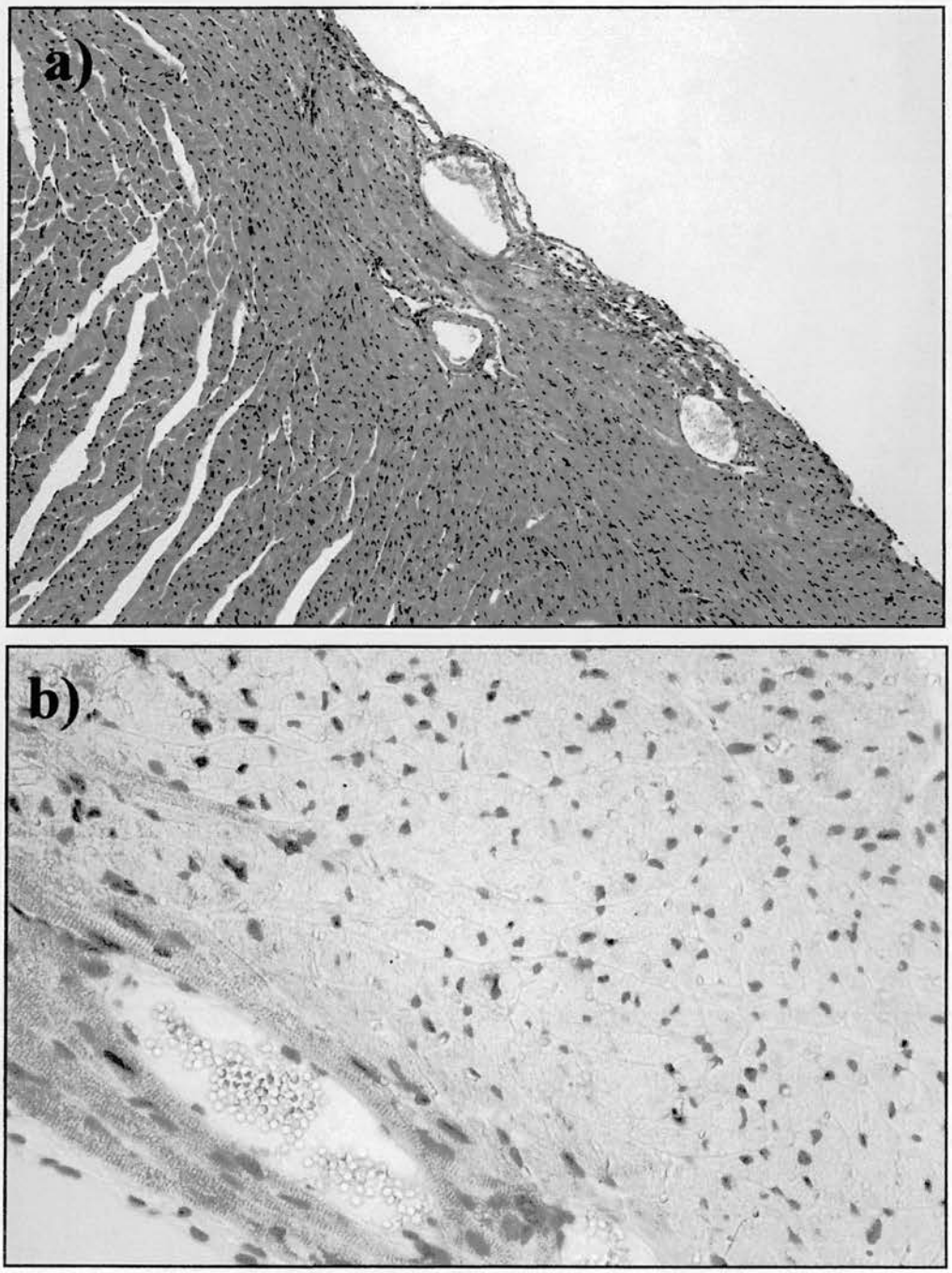
3.3.1.4.2.3 Immunoexpression of ERß following CAL in female mice

Female WT mice that had undergone CAL, in each of the oestrogen treatment groups studied, developed a collagen rich scar in the infarcted region of the left ventricle when compared to sham operated animals, *Figure 3.14* (not shown for female supplemented with oestrogen or those treated with placebo)

Immunoreactive ERß was localised to the nuclei of cardiomyocytes of sham operated animals and to the nuclei of surviving cardiomyocytes in the area of infarction, *Figure 3.14.*. Although not all of the cardiomyocytes of sham operated animals were positively stained for ERß, it appeared that surviving cardiomyocytes in the area of infarction all expressed immunoreactive ERß, *Figure 3.14.* The localisation of ERß to the nuclei of cardiomyocytes was not different in female animals that had undergone ovariectomy and administered placebo or supplemented with oestrogen (not shown).

Figure 3.14 ***Immunoexpression of ERß in the infarcted heart of female mice which are normally cycling***

Immunohistochemistry for ERß in the heart of normally cycling female mice (16- 18wks) which were either sham operated (n= 5) a) and b) or had undergone coronary artery ligation (CAL, n=8) c) and d) to induce myocardial infarction. Van Geison staining of sections of the heart indicates that a) sham operated animals did not develop an infarct whilst c) myocardial infarction occurred in mice which underwent CAL, x10 magnification. Immunoreactive ERß was detected in b) cardiomyocytes of sham operated mice and also d) cardiomyocytes in the infarcted area of CAL operated mice d) x40 magnification.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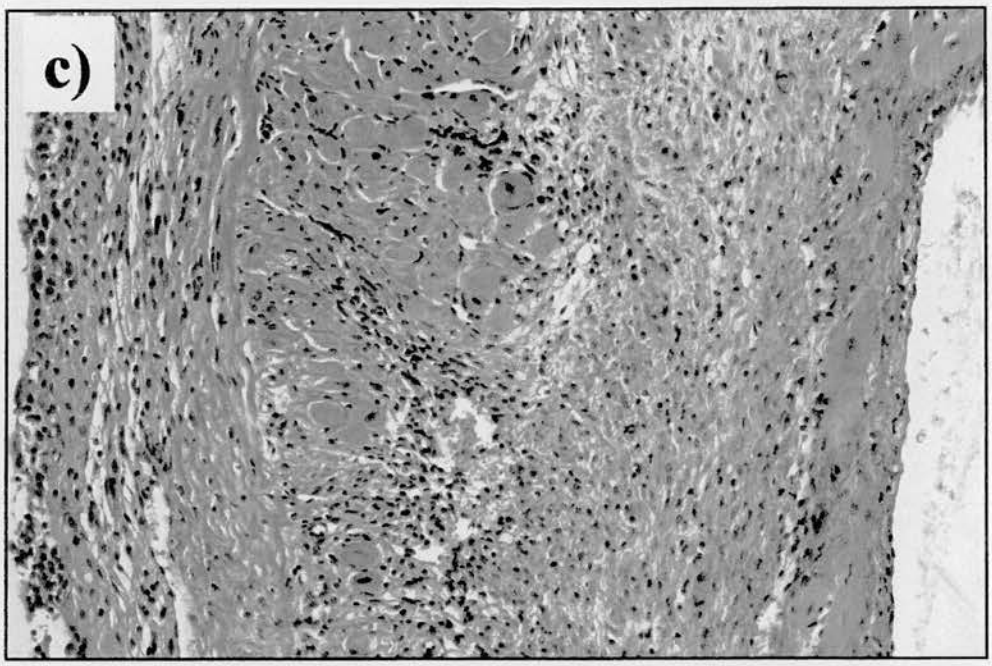
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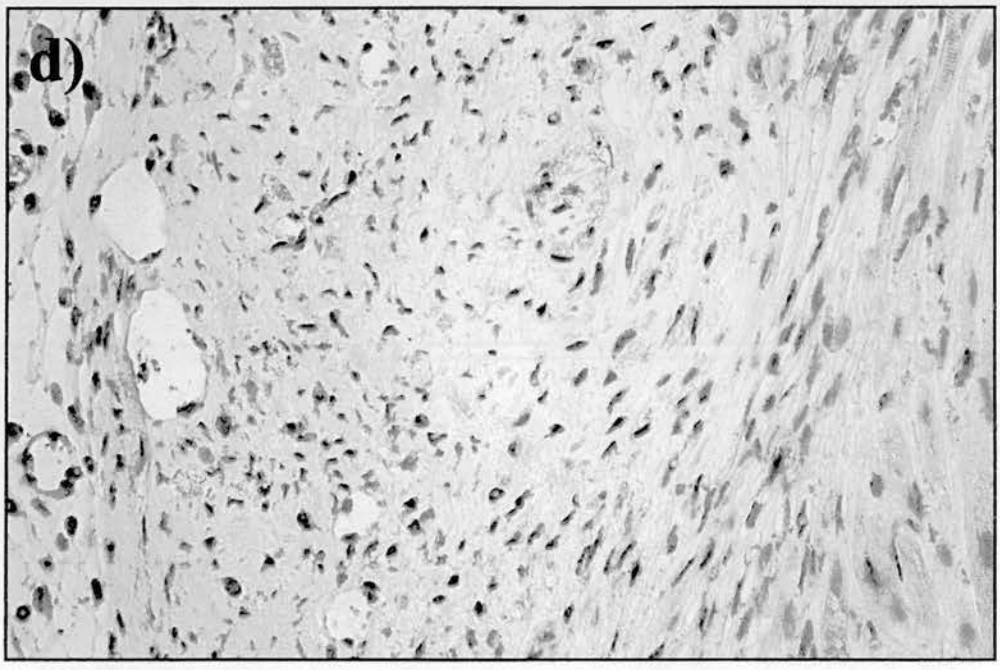
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3.4 Discussion

At the time of this study the cellular localisation and distribution of ERs in the mouse cardiovascular system was unknown. We have shown in this study that ERß was localised to the nuclei of cardiomyocytes and endothelial and vascular smooth muscle cells of aorta, mesenteric arteries and coronary arteries in both male and female WT mice. However, immunoreactive ERα was not detected in these tissues suggesting that ERß is the predominant ER isoform expressed in the mouse cardiovascular system.

We also demonstrated that immunoreactive ERß was detected in ETC and VSMC in the aorta of female atherosclerotic susceptible mice and in the region of atherosclerotic plaques that had developed in the aortic arch. This study also showed that in female WT mice, in which myocardial infarction was induced by CAL surgery, immunoreactive ERß was localised to the nuclei of cardiomyocytes in the infarcted area and also in the nuclei of cardiomyocytes in the non- infarcted region of the myocardium.

ER expression in the cardiovascular system of humans and animal models; rats, rabbits and guinea pigs (Orimo *et al*, 1993, Leiberman *et al*, 1990, Karas *et al*, 1994) has been reported to vary with the vascular bed and also species studied. In human and non-human primate, the endometrium expressed ERß but not ERα (Critchley *et al*, 2001). Although ERα was expressed in VSMC and ETC in the rat, ERα was not detected in the aorta of the guinea- pig (Orimo *et al*, 1993, Leiberman *et al*, 1990). Therefore, it was of interest to determine whether and which ERs were expressed in the cardiovascular system of the mouse and particularly those tissues in which we wanted to study the effect of oestrogen.

Our finding that ERß was expressed in the mouse heart is supported by the expression of ERß in both human and rat hearts (reviewed in Taylor and Al- Azzawi, 2000, Jankowski *et al*, 2001, Grohe *et al*, 1998). ERß expression was localised to the nuclei of

cardiomyocytes in the mouse myocardium which was similar to the expression of ERß in rat cardiomyocytes. However, a recent study by Yang *et al*, reported that expression of ERß was immunolocalised to the mitochondria of primary cultured rat cardiomyocytes (2004). This is in contrast to previous reports of ERß expression in rat cardiomyocytes and indeed our own study of expression in the mouse myocardium. It is possible that the differences between our own study and Yang *et al* (2004), is due to differences in the species studied or due to the differences in techniques used. Whereas we studied whole tissues from adult mice, in which cellular proteins were immediately fixed following tissue harvesting, they studied cultured neonatal rat cardiomyocytes and it is possible that culturing technique may alter protein expression and explain the differences between the two studies.

Interestingly, a study by Forster *et al*, reported that ERß protein was not detected in either the nuclear or mitochondrial fraction of mouse heart (2004). Although they detected several bands for ERß in the mitochondrial fraction none was specific for ERß. Attempts to establish the technique of Western blotting for the detection and quantification of ERß protein in mouse tissue in our own lab have not proven successful. However, we have clearly demonstrated in this study, through the use of two antibodies directed against different epitopes that ERß is expressed in the nuclei of cells in the myocardium in the mouse heart.

Although ERα was shown to be expressed in the rat heart (Grohe *et al,* 1998) we were unable to detect immunoreactive ERα in the mouse. It is possible that either the levels of ERα protein are below those which we are able to detect or that it is not expressed in the mouse heart. The recent study by Forster *et* al, reported that they did not detect immunoreactive ERα in the myocardium of mouse hearts however, they did not show this in their paper (2004). Further investigation is required therefore to confirm whether ERα expression in the mouse heart is either not expressed or below the level of detection, as may be suggested by expression of ERα mRNA.

Our study showed for the first time that ERß and not ERα was immunolocalised to the nuclei of VSMC and ETC of both mesenteric and coronary arteries of the mouse. Rodrigo *et al*, reported that both ERα and ERß protein could be detected by western blotting in rat mesenteric arteries (2002). Due to the differences in the expression of ERs in the vasculature between species previously reported, (Leiberman *et al*, 1990) this is not sufficient evidence to suggest that the levels are lower than the detection threshold. Although early vascular studies of oestrogen and ERs by Rubanyi *et al* reported that mRNA for ERα was detected in mouse aorta (1997), it does not necessarily follow that the message would be translated to protein. Indeed, Liang *et al* supported our finding, by reporting that ERß and not ERα was localised to the nuclei of ETCs and VSMCs in mouse aorta (2001). Therefore, the difference observed in expression of ERα between the cardiovascular system of the mice in our studies and those of rats in other studies may be attributed to differences between the species, such that ERα is not expressed in the mouse cardiovascular system. This did seem a likely explanation until later studies by Liang *et al*, reported that although they were unable to detect ERα in the mouse aorta by immunohistochemistry (2001) they were now able to detect ERα protein by Western blotting (Liang *et al*, 2003). This may be because the antibodies which are currently available for the detection of ERα are better able to detect protein which has been isolated and denatured, as with Western blotting, than those for immunohistochemistry. Therefore, it is not possible for us to conclude from our study that ERα is not expressed in the mouse cardiovascular system but rather that the levels of expression may have been undetectable by our methods, at least in the cardiovascular system as was detectable in the mouse reproductive system with the same antibody.

Subsequent chapters of this thesis studied the influence of oestrogen and the role of ERß in mediating the effects of oestrogen on the mouse cardiovascular system. As oestrogen is known to regulate the expression of its own receptors (Zou and Ing, 1998), future studies to quantify the level of expression of ERs in the mouse cardiovascular system would provide insight into the mechanisms by which oestrogen may produce its effects

on the cardiovascular system. In the vasculature, high and low concentrations of oestrogen can produce opposing effects. Interestingly a study by Rodrigo *et al*, reported that rats supplemented with oestrogen for 4 days have an almost 3 fold increase in expression of ERß protein in mesenteric artery in comparison to ovx and vehicle treated animals, whereas expression of ERα protein remained unchanged (2002). Initial stages in the development of real- time RT- PCR to quantify ER expression have been undertaken in our lab but are not yet complete. In addition to this, establishing the technique of Western blotting for ERs would allow us to relate the level of ER mRNA to the quantification of protein expressed for each receptor.

One of the aims of this thesis was to determine the role of ERß in the cardiovascular system using the ßERKO mouse as our experimental model. However, we wanted to confirm that ERß was not expressed in the tissues to be studied in these animals. As clearly shown in this chapter, ERß was immunolocalised to the heart, aorta and mesenteric arteries of ßERKO (as determined by genotyping), with a similar distribution to that of WT mice. This was confirmed using two different antibodies that were directed against different regions of the ERß protein. In order to replicate the data in the paper by Krege *et al* (1998), we used an antibody directed against the c- terminus of ERß. The ßERKO mice were prepared by insertion of a *neo* cassette into exon 3 which codes for the DNA binding domain of the receptor. This was reported to alter the reading frame of the protein resulting in a premature stop codon and therefore should not contain any of the protein coded by the exons 5- 8 (ligand binding domain). However, in our hands ERß protein was detected using the antibody directed against the c-terminus not only in tissues of the cardiovascular system but also in the ovaries from ßERKO mice in a similar expression pattern to that of the WT mouse. The second antibody used was directed against the hinge region of the protein which is immediately downstream of the DNA binding domain. ERß was localised in an identical manner to that detected using the c-terminus antibody by the antibody directed against the hinge region. Although immunoreactive ERß was detected in the ßERKO mouse we confirmed that it was unable to function as native WT protein in the ovarian follicles as ßERKO mice

presented with a similar ovarian phenotype to that described by Krege *et al* (1998). Although WT littermates were on the same mixed genetic background (C57Bl6/ SvJ129), to exclude the possibility that the phenotype observed could be explained by the genetic background of the animals, ovarian morphology of SvJ129 mice was also analysed. As the morphology of SvJ129 ovaries was similar to that of WT mice used in this study, we concluded that the phenotype observed in the ßERKO mouse could solely be attributed to the disruption of the ERß gene and subsequently the function of ERß protein expressed in these mice.

The occurrence of transcriptional read through in the generation of knock out animals, using a comparable targeting strategy, has been reported with the ERKO mouse (Couse *et al*, 1995). Indeed, it was shown in the original targeting paper of the ßERKO mouse (Krege *et al*, 1998) that alternate splicing of the ERß gene could also occur in the targeted gene, resulting in ERß mRNA variants. Two of the variants result in clones that lack exon 3 and generate stop codons in exon 4. As the antibodies used in this study were downstream of the region coded for by exon 4, it is unlikely that our colony of mice were of these variants. However, a third clone was produced from the targeting strategy which lacked the exons coding for the DNA binding domain but preserves the reading frame, such that protein downstream of the DNA binding domain would be translated. Expression of ERß isoforms lacking either exon 4 or exon 5 have been reported in both rats and humans where their function has yet to be defined but it has been suggested that they may function as dominant negative receptors (Price *et al*, 2000, Inoue *et al,* 2000). Further studies in our animals are required to determine whether the form of ERß expressed in these animals has any residual activity whilst also determining the regions of protein which are expressed in this animal following gene disruption. Western blotting of protein samples from these mice for ERß are currently ongoing to determine whether our colony of ßERKO mice express a truncated form of the ERß protein and which regions of the native WT ERß protein are missing.

This chapter also included a preliminary study to determine whether immunohistochemistry, applied to study the localisation of ERß in the mouse cardiovascular system, could be used to study the expression of ERß in mouse models of atherosclerosis and myocardial infarction. To determine the expression and localisation of ERß in atherosclerotic vessels, we used Apo E-/- mice which are a well established animal model of atherosclerosis. The literature reports that advanced atherosclerotic plaques containing extracellular lipids and fibrous connective tissue had developed in Apo E-/- by 24 weeks of age (Bentzon *et al*, 2003). The female mice used in this study were aged between 32- 36 weeks and confirmed as a suitable model for determining the expression of ERs in atherosclerosis as vessels from female mice, aged 40 weeks, were shown to contain fatty lesions in the aortic arch.

Several studies have reported that oestrogen has an atheroprotective effect in Apo E-/- mice in that it dramatically inhibited lesion initiation, and progression such that it rarely progressed beyond small uncomplicated fatty streaks consisting of macrophage foam cells (Bourassa *et al*, 1996, Elhage R *et al*, 1997, Tse *et al*, 1998). Although oestrogen has beneficial effects on lipid profile, the atheroprotective effects of oestrogen were suggested to be mediated by direct effects on the vascular wall as protection occurred at doses of oestrogen which did not alter lipid profile (Chae *et al*, 1997). Surprisingly, the localisation of ERs in the mouse model of atherosclerosis has not been characterised despite several studies using ER knock-out mice as a tool to elucidate the mechanisms by which oestrogen may mediate the apparent atheroprotection. Our initial studies demonstrated, using immunohistochemistry, that ERß was immunolocalised to both vascular smooth muscle and endothelial cells of normally cycling female Apo E-/- mice in both the aortic arch, the site of atherosclerotic lesions and in the thoracic aorta. Although there are no reports in literature to support our finding in Apo E-/- mice, Nakumura *et al* reported that ERß was expressed in human aorta containing atherosclerotic lesions (Nakumura *et al*, 2004). The study by Nakumura *et al*, also reported the expression of ERα in such lesions however we were unable to detect levels

of ERα protein by immunohistochemistry in female WT mice which thereby prevented optimisation of the conditions.

A role for both ERs in mediating the protective effects of oestrogen in atherosclerosis has been demonstrated in both human and mouse. In human vessels, ERα and ERß expression, as measured by immunohistochemistry and real- time PCR, in the neointima decreased in intensity and number in proportion to the development of atherosclerosis (Nakamura *et al*, 2004). This suggests that the reduction in the expression of ERs in the neointima may suppress the anti- atherogenic effects of oestrogen. However, whether the reduction of ERs in the atherosclerotic vessel was cause or effect remains to be elucidated. Although the expression of ERα protein has not as yet been studied in the vasculature of Apo E-/- mice, studies by Hodgin *et al*, demonstrated a role for ERα in mediating the protective effects of oestrogen through the generation of a double ERα/ Apo E-/- knock out mouse (2001). They demonstrated that oestrogen reduced lesion size by more than 80% in Apo E-/- mice which expressed ERα compared to less than 35% in mice lacking ERα. This was supported by Adams *et al*, who reported that dietary soy isoflavones reduced the levels of esterified cholesterol in the aorta of Apo E-/- mice and that the reduction in esterified cholesterol levels was lost with the loss of expression of ERα (2002). When the cholesterol efflux pathway becomes saturated, as a protective response to the increase in free circulating cholesterol levels, cholesterol is esterified (Fazio *et al*, 2001). Therefore the study by Adams *et al*, by measuring the levels of esterified cholesterol, demonstrated that ERα mediated the atheroprotective effect of oestrogen by reducing cholesterol levels (2002). However, an additional arm of the study also addressed the role of ERβ by crossing the βERKO mouse with the Apo E-/-. In contrast to the ERKO/Apo E-/-, the loss of ERβ did not inhibit the reduction in esterified cholesterol by isoflavones in dietary soy. Taken together these studies implicate a role for ERα but not ERβ in mediating the atheroprotective effects of oestrogen on plaque development. However, Hodgin *et al* suggested that ERβ may also mediate the protective role of oestrogen in plaque development as the lesions which did

develop in ERKO mice were essentially fatty streaks of lipid laden foam cells and had few advanced characteristics (2001). Therefore, following from our initial observation that ERβ is expressed in the aorta of Apo E-/- mice, we would like to investigate the effect of oestrogen on plaque development and the level of expression of ERs during plaque development. Although we were unable to optimise the technique of immunohistochemistry for ERα in the mouse cardiovascular system and subsequently unable to determine whether ERα is expressed in the vasculature of Apo E-/- mice, an alternative will be to use real- time RT-PCR. This will show whether mRNA for both ERα and ERβ is expressed in vessels from these mice and secondly, enable quantification of receptor expression. Also of interest would be the use selective ER agonists or antagonists as pharmacological tools to determine the role of each individual receptor on mediating the effects of oestrogen during plaque development.

In this preliminary study of the expression of ERβ in the mouse models of cardiovascular pathophysiology, we also examined the effect of CAL and subsequently MI on the expression of ERß in the mouse myocardium and cardiac function. We demonstrated that immunoreactive ERß was detected in the nuclei of cardiomyocytes in the infarcted region and also in the nuclei of cardiomyocytes in the non- infarcted region. However, for the reasons explained previously, we were unable to confirm whether ERα was expressed under pathophysiological conditions in the mouse heart as it appeared from our own studies that ERα expression in the mouse heart. Following myocardial infarction we demonstrated that cardiac function was impaired as assessed by ejection fraction and fractional shortening as a measure of left ventricular systolic function but that this was not different between animals of different oestrogen status. It was demonstrated that CAL led to a reduction in ejection fraction which was unaffected by oestrogen status. CAL also resulted in a reduction in fractional shortening which was not different between the three treatment groups. However, the difference in fractional shortening of placebo treated animals was not found to be significantly lower following CAL than those which were sham operated or ovariectomised and supplemented with oestrogen. However, the trend for CAL to reduce fractional shortening in placebo treated

animals may have reached significance if the number of animals studied in this group was increased, as ejection fraction was shown to be decreased in placebo treated animals and both ejection fraction and fractional shortening are measures of left ventricular systolic function. A recent study by van Eickels *et al* supported our finding that the oestrogen status of mice had no affect on impaired cardiac function post- MI (2003). However these authors did demonstrate that chronic oestrogen treatment reduced infarct size and reduced cardiomyocyte apoptosis. Several studies have suggested that the reduction in infarct size by oestrogen is receptor mediated. A possible mechanism for the reduction in infarct size due to inhibition of apoptosis following oestrogen treatment, was the finding that cardiomyocytes transfected with either ERα or ERß can inhibit p65/p50 NF-κB DNA binding complexes which would otherwise lead to the activation of genes which would lead to programmed cell death (Pelzer *et al*, 2001). Furthermore, a study in female rats which had undergone CAL and as a result developed cardiac hypertrophy reported that oestrogen treatment elevated levels of ANP (Jankowski *et al*, 2001). ANP is suggested to have anti-hypertrophic properties by attenuating protein synthesis via cGMP (Horio *et al*, 2000) and interestingly the study by Jankowski *et al*, demonstrated that ERα expression in the heart was greater than that of ERß and secondly, that the expression of ANP in the heart reflected that of ERα. This suggests that ERα may mediate the oestrogen induced increase in ANP. In further support of a role for ERα, an ERα selective agonist was recently reported to reduce hypertrophy and improve cardiac contractility in female SHR rats (Pelzer *et al*, 2004). Interestingly, the antihypertrophic effects of this drug were not blocked by an ER antagonist. However, the study did not place too much of an emphasis on this finding as they considered the cardioprotective effects mediated by the ERα agonist could be attributed to the concomitant increase in cardiac contractility as concluded from the increase in cardiac output and expression of α-myosin heavy chain. We have not as yet measured infarct size in the hearts of mice from each of the oestrogen treatment groups studied and indeed it will be interesting to determine whether, as expected from the literature, oestrogen reduced infarct size in our model also.

In mice, it was suggested that oestrogen acting through ERα in the myocardium, although it is yet not known whether ERα protein is expressed in mouse myocardium, is protective against ischaemia reperfusion injury as ERKO mice have fewer viable myocytes following such an insult than WT controls (Zhai *et al*, 2000). A more recent study by Pelzer *et al*, reported that following MI, female βERKO mice had increased mortality and biochemical markers of heart failure when compared to WT littermates (2005). Therefore from these studies it would be interesting to extend our own study from which we demonstrated that ERβ was expressed in the myocardium of infarcted hearts, to study the effects of oestrogen in our mouse model of MI and the effects of oestrogen on the expression of ERs under these pathophysiological conditions. Although we were unable to apply immunohistochemical techniques to determine the expression of ERα in the mouse heart, using real- time RT-PCR we would hope to study the expression of both ERα and that of ERβ.

In summary, this study suggests that ERß is expressed in the nuclei of cardiomyocytes, vascular smooth muscle and endothelial cells in both aortic and mesenteric vascular beds in male and female mice. Expression of ERß in the cardiovascular system was found to be unaltered under pathophysiological conditions as was detected in the nuclei of infarcted hearts and atherosclerotic vessels.

However, we were unable to detect ERα in the mouse cardiovascular system although the method was suitable for the detection of ERα as demonstrated by the expression of immunoreactive ERα in the mouse ovary. This suggests therefore, that either ERα is not expressed in the mouse cardiovascular system or that protein expression is below the levels able to be detected by immunohistochemistry.

The detection of immunoreactive ERß in ßERKO mice despite female ßERKO mice presenting with a similar ovarian phenotype to that described in the original targeting paper (Krege *et al*, 1998) suggests that the expression of residual ERß protein does not function as native WT ERß.

Chapter 4

Role of ERβ in the cardiovascular system of male mice and the effect of ageing

4.1 Introduction

In the male, oestrogen is produced by local tissue aromatisation of androgenic precursors from the testes and adrenal glands by the enzyme aromatase. Aromatase is expressed in the vasculature suggesting that there may be a local paracrine role for oestrogen in blood vessels (Simpson *et al*, 2002).

Several clinical observations and animal models report that endogenous oestrogen in males modulates endothelial function and support a role for endogenous oestrogen in the male cardiovascular system. Sudhir *et* al (Sudhir *et al*, 1997a, Sudhir *et al*, 1997b) reported that, in a young man, a deletion mutation in the ER gene lead to oestrogen insensitivity which was manifested in part, by premature coronary artery disease and endothelial dysfunction. Furthermore, studies in healthy young men, in which the enzyme aromatase was inhibited, demonstrated a reduction in flow-mediated dilation (Lew *et al*, 2003), suggesting that endogenous estrogens are involved in regulation of endothelial function in healthy males. Complimentary to this study, an animal model of male mice lacking endogenous oestrogen due to the lack of functional aromatase, had a reduced response to ACh in aortic rings supporting the view that in male mice, oestrogen facilitates vasorelaxation (Kimura *et al*, 2003).

The generation of ERα (ERKO, Lubahn *et al*, 1993) and ERß (ßERKO, Krege *et al*, 1998) knock-out mice have provided a useful tool by which to study the receptors involved in mediating the effect of oestrogen. Studies by Rubanyi *et al* reported that aortic rings isolated from male ERKO mice had a significant reduction in response to the NO synthase inhibitor, L-NAME indicating a reduction in basal release of NO relative to WT controls (1997). These results suggest that ERα in the vasculature of the male mouse has a predominant role in modulating endothelial NO production (Rubanyi *et al*, 1997).

ERß has also been implicated in mediating the effects of oestrogen in the vasculature but its role remains to be fully elucidated. Blood vessels isolated from male ßERKO

mice were found to have an enhanced relaxant response to acute administration of pharmacological concentrations of 17ß- oestradiol (Nilsson *et al*, 2000). Nilsson *et al* suggested, therefore, that ERß may modulate vascular relaxation directly or indirectly by inhibiting the activity of ERα.

The aims of this study were to further characterise the role of ERß in regulating endothelial and vascular smooth muscle cell function using the ßERKO mouse obtained through collaboration with Prof Gustafsson. At the time of planning this study, Zhu *et al* (2002) reported that ßERKO mice developed hypertension with age. Therefore, we decided to study vascular function in both young and aged animals whilst also including studies of cardiac function.

4.2 Methods

4.2.1. Animals

WT and ßERKO male mice were maintained, bred and genotyped as outlined in *Section 2.2*. To study the effect of both genotype and age, animals were assigned to one of two age groups for the study. The group of young animals were aged 14-18 weeks, WT (n= 5) and ßERKO (n= 5), and the aged group were 52- 54 weeks, WT (n= 5) and ßERKO (n= 6) upon commencing the study.

4.2.2 Analysis of cardiac function

Animals were anaesthetised and cardiac dimensions were measured by echocardiography as outlined in *Section 2.3.2.2.*, enabling cardiac function to be assessed between mice lacking functional ERß compared to age- matched WT controls. In addition, the effect of age on animals of the same genetic background could also be analysed.

4.2.3. Millar catheter blood pressure measurement

Blood pressure was measured in unconscious animals over a period of 5min once the animal had stabilised as outlined in *Section 2.3.2.1.* An average of MABP, over 5 separate time points, was calculated for each animal.

The mean arterial blood pressure was analysed to determine whether ERß influenced blood pressure in young and aged mice, by comparing WT and ßERKO mice within these age groups and also whether any influence of ERß on MABP altered with age by comparing MABP of both genetic backgrounds between age groups.

At the end of *in-vivo* analysis, tissues were harvested as outlined in *Section 2.4.1* to measure tissue weight and obtain thoracic aorta for functional analysis of the vasculature.

4.2.4. Assessment of vascular function

Thoracic aorta was removed from each animal following exsanguination, two rings of endothelium intact aorta was obtained from each animal. Aortic rings were mounted on separate chambers of the myograph and prepared for functional analysis as outlined in *Section 2.5.1.1* and *Section 2.5.2.*

4.2.4.1. Response to agonist induced contraction

To determine whether ERß was involved in regulating the contractile function of vascular smooth muscle cells and whether this altered with age, a cumulative concentration response curve (CRC) to the α_1- adrenoceptor agonist, phenylephrine (PE, 10^{-9}- 10^{-6}M) was carried out on aortic rings from male WT and ßERKO mice. Once the CRC was complete, PE was washed out and rings were allowed to re-equilibrate for 15 minutes before continuing with the experimental protocol.

4.2.4.2 Endothelium -dependent and -independent vasodilation

To investigate the response to vasodilating agents, both aortic rings were pre-constricted by a submaximal concentration of PE (~ EC_{80}) obtained from the PE CRC. In the first of the two rings, to assess endothelium- dependent relaxation and the stimulated release of nitric oxide from endothelial cells, a CRC to the muscarinic receptor agonist acetylcholine (ACh, 10^{-10}- 10^{-5}M) was carried out on aortic rings from each experimental group. To determine the ability of vascular smooth muscle cells to respond to NO, a CRC to the nitric oxide donor, sodium nitroprusside (SNP, 10^{-11}- 10^{-6}M) was performed on the second of the 2 aortic rings obtained from each animal. Once the CRC was complete, ACh or SNP, depending on the ring of aorta, was washed out and allowed to re- equilibrate for 15mins before continuing with the protocol.

4.2.4.3 Basal release of NO and constrictor prostanoids

The potentiation of the response to PE in vessels pre- incubated with the nitric oxide synthase inhibitor L-NAME (10^{-4}M, for 40min), which blocked the synthesis of NO and subsequently its release, when compared to the response to PE when L-NAME was not present, was an indication of the levels of basal NO activity in the vasculature. One of the aortic rings obtained from each animal was incubated with L-NAME and a concentration of PE that produced 80% of the maximum response to PE was used to contract the vessel.

The second aortic ring obtained from the same animal was incubated with indomethacin (10^{-5}M, for 40min) which inhibits the release of prostanoids by inhibiting the enzyme cyclooxygenase (COX). Preliminary experiments in our lab demonstrated that in this strain of mouse, which was on a mixed C57Bl6/SvJ129 background, incubation with indomethacin inhibited the synthesis and release of constrictor prostanoids from the endothelium. The response to PE (~EC_{80}) was therefore decreased by the presence of indomethacin when compared to that produced in the vessels which had not been incubated with indomethacin. It was the decrease in the response to PE by indomethacin that provided an indication of the basal levels of constrictor prostanoids.

4.3 Results

4.3.1 The effect of age and loss of functional ERß on body and tissue weight

Body weight in both WT and ßERKO mice was found to increase with age. However, there was no significant difference between genotypes, 2-way ANOVA (genotype x age) $p<0.05$, *Table 4-1*. Tissue harvested from both young and aged male WT and ßERKO mice was weighed. There were no significant difference in the weight of the heart, spleen or liver between genotype, WT and ßERKO, of young and aged mice. However, lung weight decreased with age in WT animals but not in the ßERKO, *Table 4-1*.

	Wild Type		ßERKO	
	Young	Aged	Young	Aged
Body weight (g)	31.09± 1.28	37.96± 0.51*	28.14± 0.77	42.45± 1.51*
Heart (% BW)	0.5± 0.02	0.53± 0.02	0.49± 0.01	0.51± 0.05
Lung (% BW)	0.63± 0.01	0.58± 0.02*	0.59± 0.01	0.57± 0.02
Spleen (% BW)	0.32± 0.02	0.27± 0.04	0.29± 0	0.26± 0.02
Liver (% BW)	4.71± 0.16	4.62± 0.23	4.59± 0.13	4.18± 0.2

Table 4-1 ***Body and tissue weights for WT and ßERKO mice***

*Young (14- 18wks) and aged (52- 54 wks) WT and ßERKO mice were weighed and tissue harvested was weighed and expressed as a percentage of body weight (BW). Values are expressed as mean ± SEM and data analysed using a 2- way ANOVA (genotype x age) followed by a Bonferroni post hoc test, *p<0.05 compared to young cohort of animals (n=5 in each group, except for aged ßERKO, n= 6).*

4.3.2. Role of ERß on blood pressure and the effect of age

MABP was measured in both WT and ßERKO, young and aged male mice, *Figure 4-1*. There was no significant difference in mean arterial blood pressure (MABP) between age- matched WT and ßERKO animals in either the young or aged cohort of animals studied. There was a significant increase in MABP with age in both WT and ßERKO animals, *Figure 4-1*.

4.3.3 Effect of loss of functional ERß and age on cardiac function

Cardiac dimensions were measured in both WT and ßERKO male mice which subsequently enabled analysis of cardiac function. As shown in *Figure 4-2* the ejection fraction of young ßERKO male mice was significantly greater than that of age-matched WT littermates. However, this difference in ejection fraction was no longer found to be significant between aged WT and ßERKO animals.

Although ejection fraction increased in mice lacking functional ERß when compared to WT littermates, there was no difference in fractional shortening observed either with genotype or age, young; WT 0.31± 0.02, ßERKO 0.33± 0.01, aged; WT 0.38± 0.01, ßERKO 0.32± 0.04, 2- way ANOVA (genotype x age).

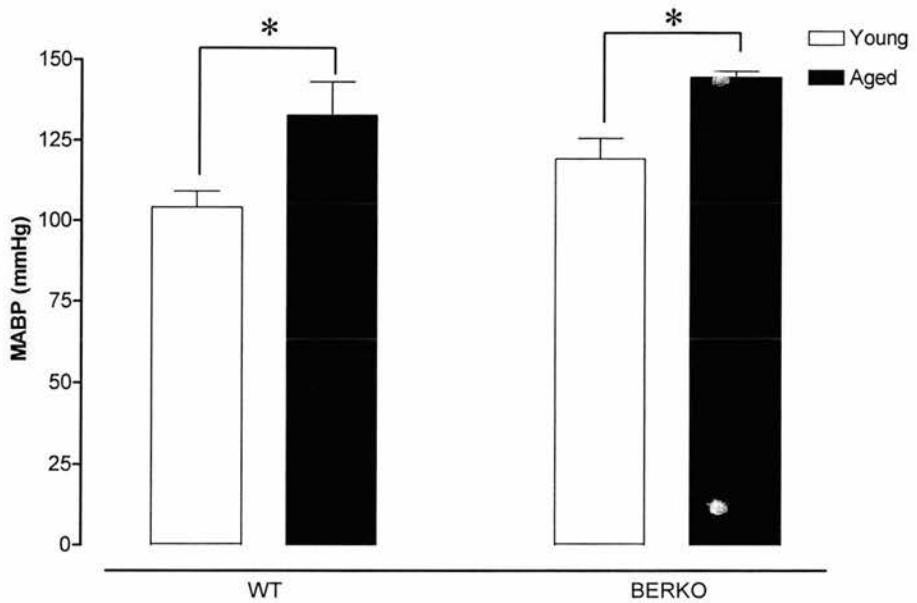

Figure 4-1 ***MABP in both young and aged WT and βERKO mice***

*MABP of anaesthetised young (14- 18wks) and aged (52- 54wks) WT and βERKO mice measured by carotid cannulation. Data are expressed as mean± SEM Data was analysed using a 2 way-ANOVA followed by a Bonferroni post- hoc test, *p<0.05 (n= 5 in each group except for aged βERKO, n= 6).*

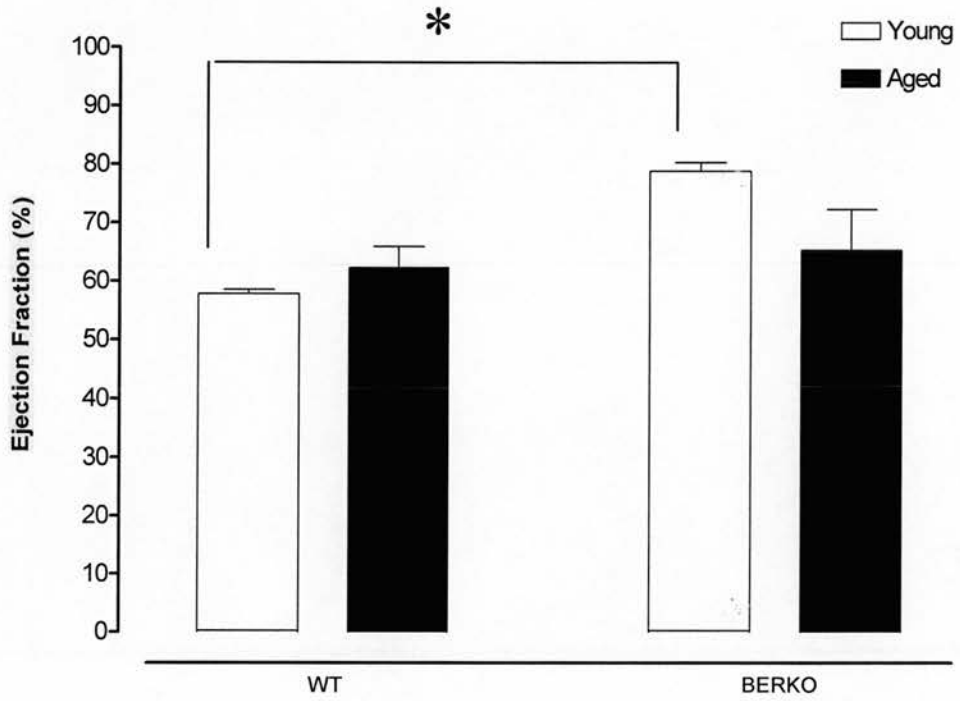

Figure 4-2 ***Assessment of ejection fraction in male mice***

*Ejection fraction measured in both young (14- 18wks) and aged (52-54wks) WT and βERKO male mice. Data are expressed as mean± SEM and analysed using a 2-way ANOVA (genotype x age) followed by a Bonferroni post- hoc test. *p<0.05 (n=5 in each group, except for aged βERKO, n= 6)*

4.3.4 Analysis of vascular function

4.3.4.1 Influence of ERβ on agonist induced contraction of vascular smooth muscle cells.

Cumulative addition of PE to aorta isolated from both young WT and ßERKO mice lead to contraction of the vascular smooth muscle, *Figure 4-3*. The response to PE was expressed as a percentage of the response to 60mM KCl which was similar between all groups, young; WT 1.73± 0.13g, βERKO 1.35± 0.2g, aged; WT 1.46± 0.11g, βERKO 1.31± 0.18g, 2- way ANOVA (genotype x age).

The vasculature of mice lacking functional ERβ had increased sensitivity and higher E_{max} to PE than their age- matched WT controls *Figure 4-3a* and *Table 4-2*. The response to PE was not different between young and aged WT mice, *Table 4-2,* in that neither the EC_{50} nor E_{max} were significantly different. Although the sensitivity to PE in vessels from aged βERKO mice was greater than that of age matched WT controls, *Figure 4-3b,* the sensitivity to PE was not altered by age in the βERKO mouse, *Table 4-2*.

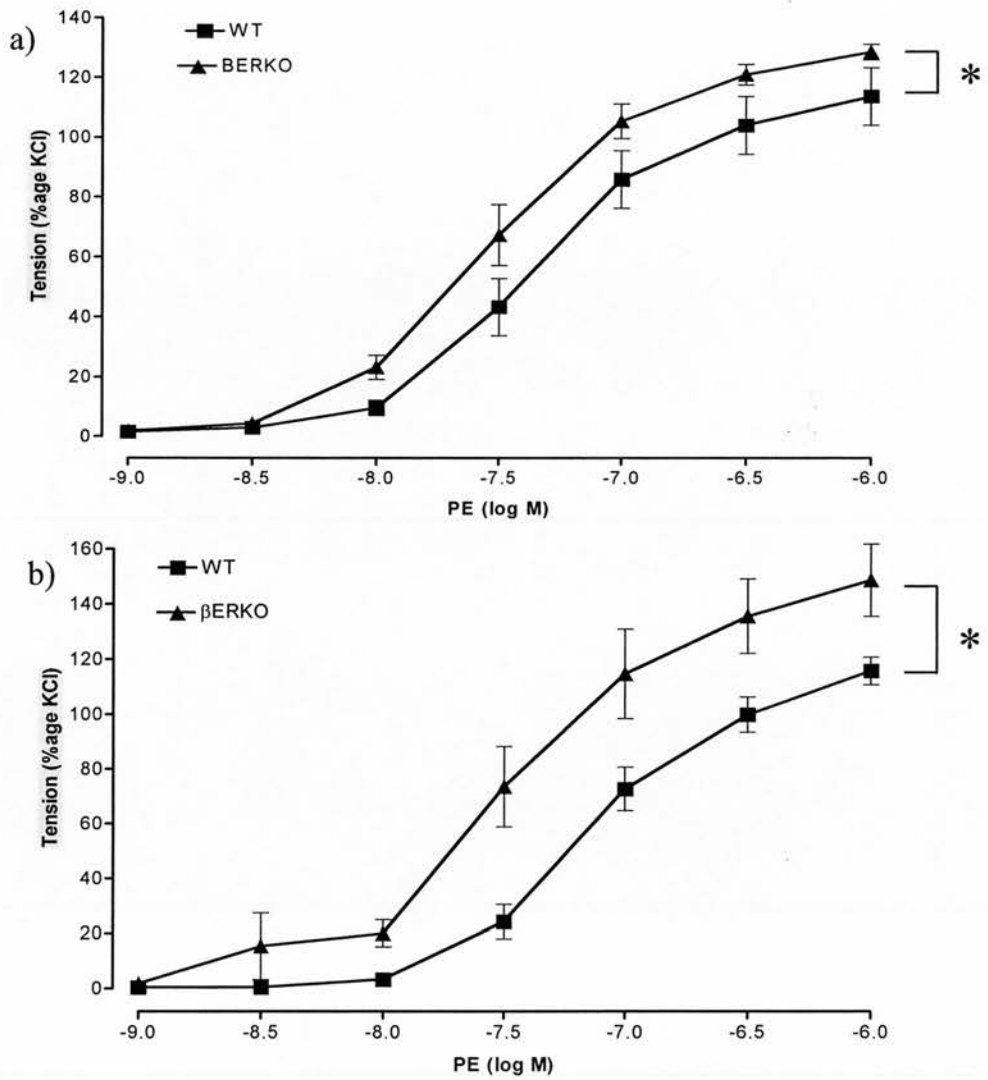

Figure 4-3 ***Response to PE in the vasculature of male mice***

*Cumulative concentration response to PE (10^{-9}- 10^{-6}M) in aortic rings with intact endothelium isolated from a) young (14- 18wks) WT (■, n=5) and βERKO (π, n= 5) and b) aged (52- 54wks) WT (■, n=5) and βERKO (π, n= 6) . Values expressed as mean± SEM and analysed using a 2- way ANOVA (genotype x age) *$p<0.05$.*

	WT		βERKO	
	Young	Aged	Young	Aged
PE EC_{50} (M)	$6.7x10^{-8}\pm$ $2.2x10^{-8}$	$8.5x10^{-8}\pm$ $2.0x10^{-8}$	$2.8x10^{-8}\pm$ $6.2x10^{-8}$*	$3.7x10^{-8}\pm$ $2.1x10^{-8}$*
PE E_{max} (%KCl)	113.81± 9.67	115.73± 4.97	128.76± 2.61*	148.71± 13.13*

Table 4-2 ***Contractile responses in the vasculature of WT and βERKO mice***

The EC_{50} and maximal contraction (E_{max}) were calculated for the contractile effect of PE in isolated, endothelium intact aorta from young (14- 18wks) and aged (52- 54wks) WT and βERKO mice. Data are mean± SEM of n = 5 in each group, except for aged βERKO, n= 6. Statistical difference ($p< 0.05$) was analysed between age matched male WT and βERKO mice using a 2- way ANOVA (genotype x age)*

4.3.4.2 Influence of ERβ on the basal release of NO and constrictor prostanoids from the vascular endothelium

In young animals, the increased response to PE in the presence of L-NAME was less in the vasculature of the ßERKO mouse relative to their age- matched WT controls *Figure 4-4*, 2-way ANOVA (genotype x age) followed by a Bonferroni post hoc test. However, there was no significant difference between aged WT and ßERKO in the contractile response to PE in the presence of L-NAME. As there was no difference within genotype with age, it is difficult to determine whether the levels of basal NO in WT were reduced with age or that they were increased in the aged ßERKO, *Figure 4-4.*

In addition to the activation of second messenger pathways in vascular smooth muscle cells, the contractile response to PE in the mouse vasculature is also attributed to the release of constrictor prostanoids from the endothelium The reduction in the response to PE in the presence of indomethacin, an inhibitor of COX, was indicative of the levels of constrictor prostanoids released from the vascular endothelium. The response to PE in the presence of indomethacin was reduced by an equal extent in both WT and ßERKO in both age groups studied, 2-way ANOVA (genotype x age), *Figure 4-5.*

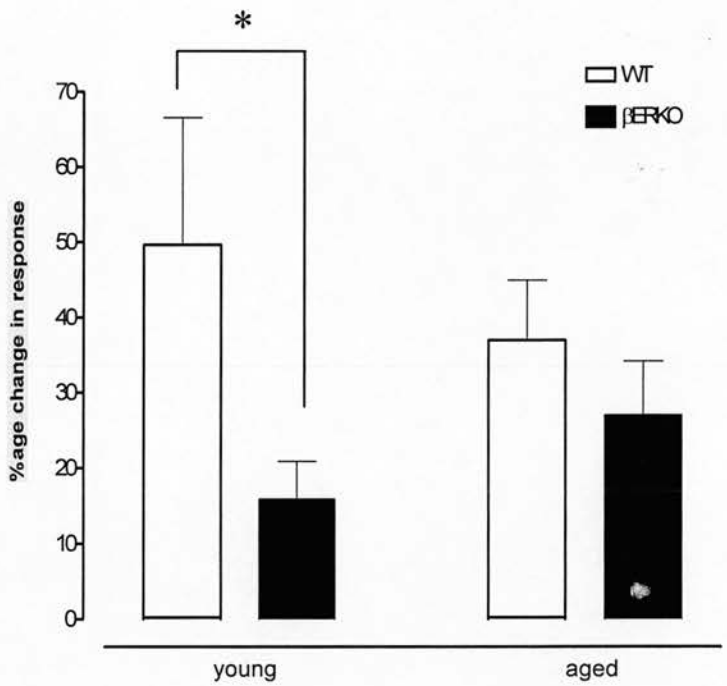

Figure 4-4 ***Basal release of NO in the vasculature of WT and βERKO mice***

*Enhancement of the contractile response to PE (~EC_{80}) in the presence of the NOS inhibitor, L-NAME (10^{-4} M). Data expressed as mean± SEM, n=5, except for aged βERKO, n= 6, and analysed using a 2-way ANOVA *p<0.05*

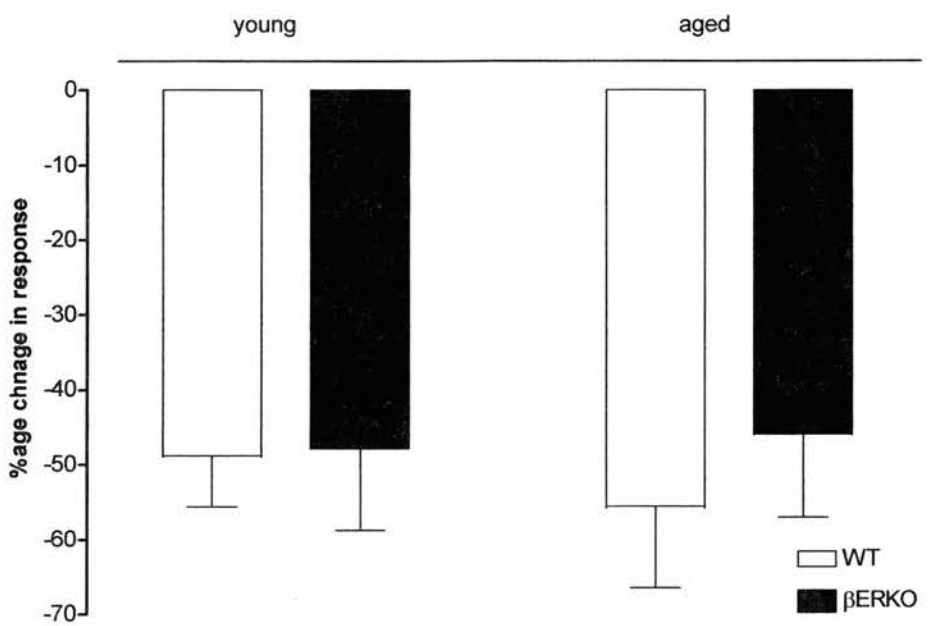

Figure 4-5 ***Basal release of constrictor prostanoids in the vasculature of WT and βERKO mice***

Reduced contractile response to PE (~EC_{80}) in the presence of the COX inhibitor, indomethacin (10^{-5}M). Data expressed as mean± SEM, n=5, except for aged βERKO, n= 6, and analysed using a 2-way ANOVA

4.3.4.3 Influence of ERß on endothelium- dependent and –independent vasodilation

4.3.4.3.1 Influence of ERß on endothelium- dependent vasodilation

Aortas from both young WT and ßERKO mice were preconstricted with PE (~EC_{80}) before constructing a CRC to either ACh or SNP.

Endothelium intact vessels from young ßERKO mice were less sensitive to relaxation induced by ACh and there was also a reduction in the maximum relaxation relative to that of age matched WT controls, *Figure 4-6a.* Age did not alter the relaxation response to ACh in WT animals, *Table 4-3.* In contrast, in the ßERKO, the E_{max} of ACh was slightly greater in aged animals relative to young, *Table 4-3.* However, similar to that observed in the young cohort of animals, vessels from mice lacking functional ERß were less sensitive to ACh and had a lower maximal relaxation response relative to age matched WT controls, *Figure 4-6b.*

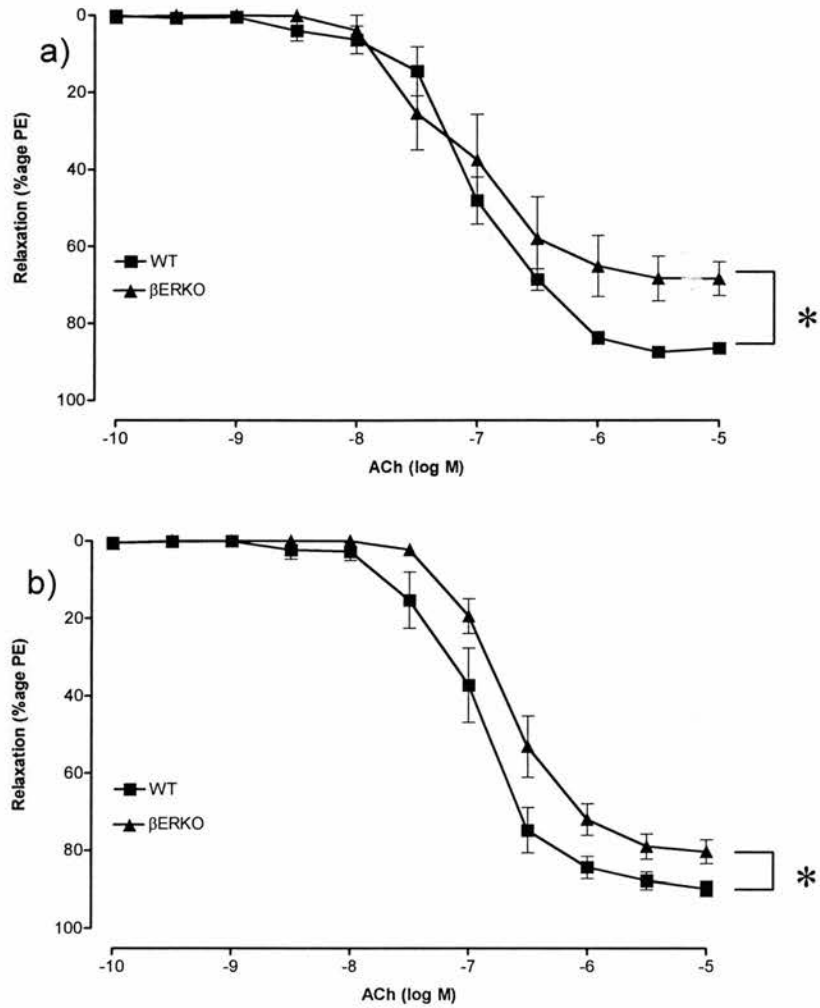

Figure 4-6 ***Relaxation response to ACh in the male vasculature***

*Cumulative concentration response to ACh in aortic rings with intact endothelium isolated from a) young (14- 18wks) WT (■, n= 5) and βERKO (π, n= 5) and b) aged (52- 54wks) WT (■, n= 5) and βERKO (π, n= 5) . Values expressed as mean± SEM and analysed utilising a 2- way ANOVA (concentration x genotype) *p<0.05*

	WT		βERKO	
	Young	Aged	Young	Aged
EC_{50} (M)	$15.0x10^{-8}\pm$ $3.1x10^{-8}$	$15.4x10^{-8}\pm$ $3.6x10^{-8}$	$41.0x10^{-7}\pm$ $2.0x10^{-7}$*	$37.0x10^{-7}\pm$ $1.0x10^{-7}$*
E_{max} (%)	86.5± 0.7	89.8± 1.9	68.4± 4.3*	80.3± 3.1*$

*Table 4-3 The **EC_{50} and E_{max} for responses to ACh in the male vasculature***

*The EC_{50} and E_{max} for ACh in intact aortic rings isolated from young (14- 18 wks) and aged (52- 54wks) WT and βERKO mice. Data are mean ± SEM of n = 5 in each group. Statistical difference was indicated between age matched male WT and βERKO mice *$p<0.05$ and with age within WT and βERKO mice $^{\$}p<0.05$ using a 2- way ANOVA (genotype x age).*

4.3.4.3.2 Influence of ERß on endothelium –independent vasodilation

The ability of VSMCs to relax in response to NO was assessed by the relaxation response to the nitric oxide donor drug, SNP, in the mouse vasculature. In young animals, the response to SNP was not found to be significantly different between ßERKO mice and age- matched WT controls, *Figure 4-7* and *Table 4-4*. The relaxant response to SNP was not affected by age in the vasculature of either WT or ßERKO mice and nor was there any difference between the vasculature of the ßERKO mice and age-matched WT controls, *Table 4-4*, 2-way ANOVA (genotype x age) followed by a Bonferroni post- hoc test.

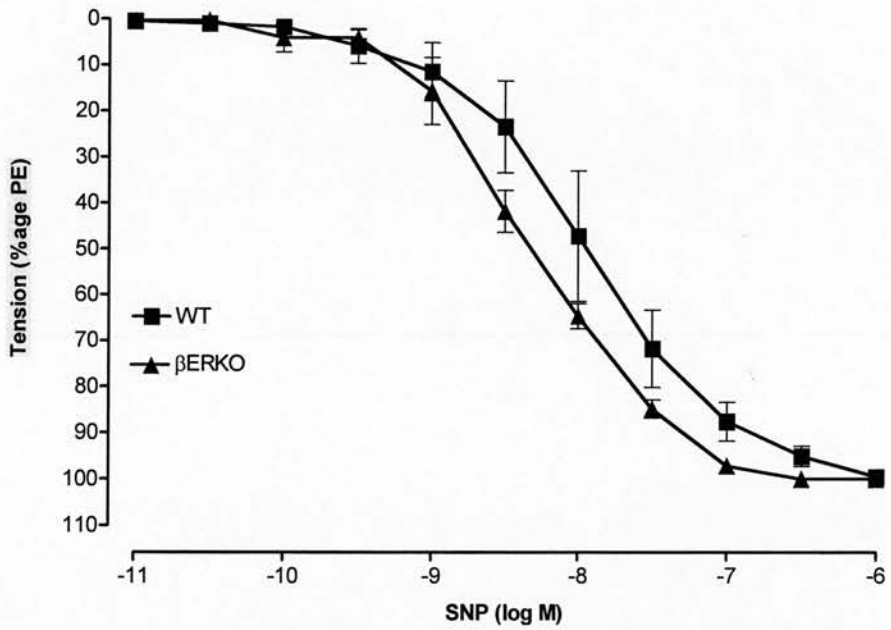

Figure 4-7 ***Relaxation response to SNP in the vasculature of male mice***

Cumulative concentration response to SNP in aortic rings with intact endothelium in vessels, preconstricted with PE (~EC_{80}), isolated from WT (■, n=5) and βERKO (π, n= 5). Values expressed as mean± SEM and analysed utilising a 2-way ANOVA

	WT		ßERKO	
	Young	Aged	Young	Aged
EC_{50} (M)	$15.6x10^{-9}\pm$ $6.8x10^{-9}$	$14.0x10^{-9}\pm$ $4.8x10^{-9}$	$4.8x10^{-9}\pm$ $1.3x10^{-9}$	$7.4x10^{-9}\pm$ $1.75x10^{-9}$
E_{max} (%)	99.5± 0.25	97.5± 1.16	100± 0.0	98.6± 1.36

Table 4-4 The ***EC_{50} and E_{max} for the responses to SNP in the vasculature of male mice***

The EC_{50} and E_{max} for SNP in intact aortic rings isolated from young (14- 18wks) and aged (52- 54wks) WT and βERKO mice. Data are mean ± SEM of n = 5 in each group, except for aged βERKO, n= 6. Data was analysed using a 2- way ANOVA (genotype x age).

4.4 Discussion

The main findings of this study were that *in- vitro* analysis of vascular function demonstrated that isolated aorta from ßERKO mice had an increased sensitivity to PE compared to age- matched WT controls. In the presence of L-NAME the increase in contractile response to PE was less in young ßERKO mice than that of age-matched WT controls. This suggests that the basal release of NO from the endothelium was reduced in animals that did not express ERß. However, in aged animals there was no difference in the basal release of NO between WT and ßERKO mice. Similar to the reduction in basal release of NO in young ßERKO mice, the stimulated release of NO, and other EDRFs, by ACh was reduced in ßERKO animals relative to WT controls. In contrast however, the difference in the response to ACh between these groups was also observed in aged animals. Although the basal and stimulated release of NO was impaired in the ßERKO mouse, the ability of vascular smooth muscle cells to relax in response to NO was the same as that of age- matched WT controls.

Despite the observed vascular phenotype in both young and aged ßERKO mice and the contribution of the vasculature to the regulation of blood pressure, there was no difference in MABP between ßERKO mice and their age- matched WT controls. In addition to assessing vascular function and blood pressure in the ßERKO mouse we also assessed cardiac function. We found that in the young cohort of animals studied the loss of functional ERß was associated with an increase in ejection fraction but not fractional shortening.

A role for ERß in the vasculature was demonstrated by our study as the sensitivity and maximum response to PE was greater in ßERKO mice than that of their WT controls. In addition, basal and stimulated release of NO was also reduced in the ßERKO. Therefore, due to the observed differences in the NO pathway in the ßERKO mouse relative to WT, it seems that the reduction in NO levels and or activity might explain the potentiation of the contractile response in the ßERKO mouse.

Chronic oestrogen treatment has been shown to increase basal release of NO in rat and rabbit aorta and as well as stimulated release of NO in rat vasculature (Paredes-Carbajal *et al*, 1995, Hayashi *et al*, 1992, Wu *et al,* 2000). The suggested mechanism by which oestrogen increases NO is by altering the activity of the enzyme which produces NO, eNOS (Gonzales *et al* 2001). However, the role of the individual receptors in regulating vascular tone has only recently begun to be studied through the use of ERKO and ßERKO mice and more recently selective ER agonists and antagonists. The contractile response to PE in the presence of L-NAME was reduced in aorta from male and female ERKO mice when compared to WT control, which suggests that the basal release of NO was reduced (Rubanyi *et al,* 1997) in mice lacking ERα. A suggested mechanism for ERα activation of NO is that eNOS is activated following the localisation of ERα to caveloae and activation of the MAPkinase pathway (Chen *et al*, 1999, Garcia-Cardena *et al*, 1998). Further studies involving ERKO and ßERKO mice lead the authors to suggest that oestrogen acting through ERα, but not ERß, increased the basal release of NO (Darblade *et al*, 2002). However, there does lie some controversy in the literature as more recent studies have demonstrated that ERß also influences the relaxation induced by NO in blood vessels. Through the recent development of ER specific agonists, Montgomery *et al* reported that although the magnitude of relaxation to ERα agonists in rat mesenteric arteries was greater than that of the ERß agonist, ERß was still clearly able to induce relaxation (Montgomery *et al,* 2003). The mechanisms by which oestrogen may increase NO through ERß are poorly understood at the moment. However, ERβ, similar to ERα, was shown to localise to functional signalling domains, caveolae, where it mediated non- genomic activation of eNOS that was independent of ERα (Chambliss *et al*, 2002). In addition, Zhu *et* al reported that loss of ERß inhibited the ability of oestrogen to attenuate constriction in mouse aorta in which the endothelium had been removed (Zhu *et al*, 2002). The authors suggested that this was due to the reduction in oestrogen induced iNOS expression in the ßERKO mouse reported in this study.

Oestrogen, which because it has a hydroxyl group on its phenolic A- ring, is shown to possess natural antioxidant effects similar to vitamin E by suppressing free radical

induced peroxidation chain reactions (Sugioka *et al*, 1987, Green *et al*, 1997). These antioxidant effects are achieved by improving NO/ O_2^- balance at a genomic level by inhibiting a subunit of NADPH oxidase, gp91phox, which is an important source of O_2^- and thereby reduce the cells capacity to generate O_2^- (Wagner *et al*, 2001). More recent studies have implicated a specific role for ERß in mediating the antioxidant effects of oestrogen. Tamir *et al* reported that the generation of reactive oxygen species within physiological concentrations was sufficient to increase ERß expression whilst ERα remained unchanged in several cell types (2002). Furthermore, Montano *et al*, demonstrated that ERß mediated regulation of the enzymes involved in the generation of glutathione, which detoxifies electrophiles (Montano *et al,* 2004). Therefore, it seems possible that the impaired endothelial function of ßERKO mice in our study, may also, in part, be due to the loss of the antioxidant effects of oestrogen via ERß. Therefore, in mice lacking functional ERß, oxidation and subsequent inactivation of NO would be enhanced.

Preliminary experiments in our lab showed that the vasculature of the mice studied released predominantly constrictor prostanoids from the endothelium as the contractile response to PE was significantly reduced in the presence of indomethacin. There was no difference in the contractile response to PE in the presence of indomethacin between the experimental groups therefore it is unlikely that the potentiated PE response was due to an effect mediated by ERβ on the cyclooxygenase pathway. This is despite reports that oestradiol decreases cerebrovascular tone in rats by shifting the products of the endothelial cyclooxygenase pathway from constricting prosanoids, such as PGH_2 to the vasodilator prostacyclin, PGI_2 (Ospina *et al,* 2003). Other possible mechanisms for the enhanced response to PE in the ßERKO mouse could be due to a direct effect of ERß on vascular smooth muscle cells to alter ion flux, expression and or activity of receptors, or reduce the sensitivity of contractile proteins.

Literature does support a role for oestrogen in regulating ion flux as it was reported that oestrogen can enhance potassium efflux by binding to the ß1 subunit of the Maxi- K channel (Valverde *et al*, 1999, Benkusky *et al*, 2002). Subsequently these channels become more sensitive to voltage and calcium and enhance potassium

efflux. Zhu *et al* reported that the loss of functional ERß prevented the outward flux of potassium from BKca channels or Kv potassium (Zhu *et al,* 2002). Therefore, it is possible that the enhanced contractile response to PE in our colony of ßERKO mice, in addition to a reduction in NO release, can also be explained by the impaired function of K^+ channels such that K^+ efflux would be impaired and vascular tone increased.

Another possibility is that oestrogen acting through ERß alters adrenoceptor ativity and/ or expression. Chronic oestrogen treatment reduced α_1/ß- adrenoceptor- induced stimulation of melatonin synthesis and release by inhibiting ß- adrenoceptor induced accumulation of cAMP and α_1- adrenoceptor induced phosphoinositide hydrolysis (Hernandez- Diaz *et al*, 2001) Therefore, the enhanced contractile response to PE in the vasculature of animals lacking functional ERß could be explained by oestrogen acting through ERß to inhibit the hydrolysis of phosphoinositide to inositol triphosphate, thereby attenuating the release of stored calcium and subsequently, the contraction of vascular smooth muscle cells. Further vascular analysis studying the contractile response to different contractile agents, such as 5-HT or U46619, thromboxane analogue, may provide some useful insight into whether the enhanced contractile response is unique to the modification of contraction induced by α_1-adrenoceptors.

We demonstrated that MABP was not different between WT and ßERKO mice and that, as expected MABP increased with age (reviewed in Luscher *et al*, 1995 and Marin *et al*, 1995), which was found to be similar in both groups. This suggests that either ERß is not involved in the regulation of blood pressure or that ERα may compensate for the loss of functional ERß. Several studies have indicated that when both ERs are present they may have opposite effects on gene transcription but in the absence of the other, they can partially replace the transcriptional effects (Lindberg *et al*, 2003, Weihua *et al,* 2000). Indeed studies looking at the role of ERs in vascular injury observed that oestrogen provided a degree of protection in both ERKO and ßERKO mice suggesting that oestrogen may be able to act through either of the two ERs to provide the same degree of protection (Iafrati *et al* 1997, Karas *et al*, 1999). It

is possible that in the knock-out, the remaining ER expression and function is increased to compensate for the loss of the other and therefore the expression of one ER is sufficient to provide protection. However, ERβ was thought to influence blood pressure as a clinical study of post- menopausal Japanese women reported that a repeat polymorphism in the ERß gene was associated with hypertension (Ogawa *et al*, 2000). Our finding that there was no difference in MABP between aged WT and ßERKO animals was in contrast to a recently published study (Zhu *et al*, 2002) which reported that although WT and ßERKO mice both developed an increase in blood pressure with age, the increase was significantly greater in year old ßERKO mice when compared to age- matched WT mice. Possible explanations for this discrepancy was firstly, that the ßERKO mice established in our lab expressed immunoreactive ERß, refer to *Section 3.3.1.3.* Therefore the ßERKO mouse that we studied may be a variant of the ßERKO mouse generated in the original targeting paper (Krege *et al,* 1998) and indeed the ßERKO mouse used by Zhu *et al*, (2002). Further evidence to support this was from personal communication with J- A Gustafsson, he reported that the spleen and hearts of aged ßERKO mice were grossly enlarged when compared to age-matched WT controls. However, the spleen from the strain of ßERKO mice established in our lab from those generated by Krege *et al,* (1998) did not become enlarged with age when compared to WT littermate controls. Although we detected an increase in blood pressure in both aged WT and ßERKO mice when compared to young animals there were no differences in heart weight indicating that the increase in blood pressure had not led to hypertrophy of the heart, or indeed that there were any differences in heart weight between WT and ßERKO mice. Secondly, the method used for the measurement of blood pressure also differed. In this study we measured blood pressure acutely in anaesthetised animals, whereas Zhu *et al*, implanted radiotelemetry probes and measured blood pressure over an eight day period. As telemetry blood pressure studies allow the animal to recover from surgery before measurements are taken, therefore the effect of anaesthetic is removed and without the stress of handling the animal, small changes in blood pressure are more reliably detected. In the study by Zhu *et al*, year old ßERKO mice had a significant increase in blood pressure of only 10mmHg relative to age- matched WT controls. MABP in our study of aged WT and ßERKO mice was

of a similar magnitude, WT; 132.8± 10.3mmHg and ßERKO; 144.6± 1.8mmHg. Therefore, it is possible that as we measured blood pressure acutely by carotid cannulation where blood pressure will be influenced by anaesthesia and surgery to an extent, that any small differences in blood pressure between ßERKO and WT animals in our study would not reach significance due to the variability within the group.

As clearly shown in *Section 3.5* ERß was expressed in cardiomyocytes of the mouse heart. Although literature supports a role for oestrogen on the myocardium, the specific role of ERs has not been elucidated to date. Clinical studies clearly demonstrate a role for oestrogen on the heart, as they report that healthy women have higher ejection phase indices than age- matched men. In addition premenopausal women have a higher pressure- volume ratio, ejection fraction and ejection rates when compared to post- menopausal women (Buonanno *et al*, 1982, Pines *et al*, 1992). Animal studies support the clinical observations in that papillary muscles from female rats had higher rates of shortening than those of males (Capasso *et al*, 1983)

From our own studies, it appears that endogenous oestrogen in male mice may decrease left ventricular systolic function via ERß in young animals as ejection fraction was higher in ßERKO animals than age- matched WT controls and that this effect was lost with age. Interesting, however, was that the ejection fraction had increased and yet fractional shortening, another indicator of left ventricular systolic function remained unchanged and therefore it is difficult to conclude that the loss of functional ERß in the ßERKO mouse lead to an increase in systolic function. In addition, clinical studies have reported that although chronic oestrogen treatment may lead to a reduction in left ventricular wall mass in hypertensive postmenopausal women, systolic function, as measured by left ventricular fractional shortening was not modified (Modena *et al,* 1999). However, acute infusion of 17ß- oestradiol in both male and female rats increased cardiac output and the ejection fraction (Beyer *et al*, 2001). It is difficult to extrapolate a mechanism for the effect of oestrogen on the myocardium as systolic function measured by echocardiography, expressed as

ejection fraction and fractional shortening are not independent of pre- and after- load. Young ßERKO male mice had an increased response to PE and a reduction in the basal and stimulated release of NO, both of which would contribute to increase afterload. Therefore, it is more likely that the increased ejection fraction is due to a direct effect on the myocardium as increased afterload would act to reduce the ejection fraction.

One possible explanation for the increase in ejection fraction could have been cardiac hypertrophy with or without cardiac chamber dilatation. However, analysis of heart weight in our colony of animals clearly showed that there was no difference between WT and ßERKO mice suggesting that the hearts from ßERKO mice were not hypertrophic. It may also have been of benefit to increase the sample size in each of our experimental groups due to the limitations of echocardiography as a means to access systolic function. Mainly because the measurements taken are not necessarily of the same sample section of the heart between animals and that it assumes that the heart is cylindrical in shape.

In conclusion it has been shown that ERß mediates the effects of oestrogen on the male vasculature as the loss of functional ERß lead to enhanced contractility and impaired endothelial function. However, the increase in vascular tone and impaired endothelial vasodilatation were not implicated in the regulation of MABP, as this was not different between age-matched WT and ßERKO mice. Finally, the loss of functional ERß increased ejection fraction independently of fractional shortening and cardiac chamber dilation or hypertrophy.

Chapter 5

The influence of oestrogen and the effect of the proprietary, Organon oestrogen receptor ß selective antagonist on blood pressure and vascular function of the female mouse.

5.1 Introduction

Oestrogen was suggested to have a protective role in the cardiovascular system as women suffered less from cardiovascular disease during their reproductive life than age-matched men (Benetos *et al*, 1999). Furthermore, this apparent protection gradually declined after the menopause.

One such observation of the beneficial effect of oestrogen in the cardiovascular system was that it may be involved in the regulation of blood pressure. It was suggested that oestrogen lowered blood pressure as premenopausal women had a lower systolic and diastolic blood pressure than postmenopausal women (Stassen *et al,* 1989, Seely *et al* 1999, Cagnacci *et al* 1999), or women who had undergone surgical hysterectomy and ovariectomy. In addition young women have lower systolic blood pressure than age-matched men (Pearson *et al* 1997, Anastos *et al* 1991). Following the menopause not only does blood pressure increase in women but it is also typically higher than that of age-matched men (Pearson *et al*, 1997). From such observations, it was thought that HRT may have beneficial effects on blood pressure. However, clinical studies have been contradictory, Staessen *et al* (1989) reported that treatment of postmenopausal women had no effect on blood pressure, whereas, in contrast, a similar study by Scuteri *et al* (1999) reported that women taking HRT had lower blood pressure than those who were not. The PEPI trial, in which normotensive postmenopausal women were administered HRT with a 3 year follow up, showed no benefit in comparison to placebo treated women regarding blood pressure. However, a more recent study by Mohle-Boetani *et al* which had a longer follow up period than that of the PEPI trial, almost double, showed that there was a smaller increase in blood pressure in post-menopausal women on HRT than those who were not (2001). The main difference between these studies was that the age group studied were older, suggesting that the effect of the therapy on blood pressure was intensified with age.

To date the underlying mechanism(s) by which oestrogen may modulate blood pressure are not yet fully understood. Oestrogen has been reported to influence many

of the factors that are involved in the regulation of blood pressure such as autonomic tone and baroreceptor reflex. In the central nervous system, oestrogen was reported to enhance parasympathetic tone, attenuate sympathetic tone and enhance baroreceptor reflex activity (Saleh *et al* 2000, Saleh *et al*, 1998, 1999 and 2000). Oestrogen also has effects on the renin angiotensin system, where it has reportedly reduced circulating levels of renin, angiotensin converting enzyme and downregulated the expression of the AT_1 receptor (Harrison- Bernard *et al*, 2003, Roesch *et al*, 2000, Nickenig *et al*, 1998).

Oestrogen may also influence blood pressure by modulating vascular reactivity and endothelial function. Chronic oestrogen treatment increased basal release of NO in rat and rabbit aorta, as well as stimulated release of NO in rat vasculature (Paredes-Carbajal *et al*, 1995, Hayashi *et al*, 1992, Wu *et al*, 2000). Although the exact mechanism by which oestrogen increases NO is as yet unclear, a suggested mechanism was that oestrogen alters the activity of the enzyme, eNOS, which is involved in the production of NO (Gonzales *et al*, 2001).

The role of individual ERs in regulating blood pressure and vascular tone has only recently begun to be studied through the generation of ERKO and ßERKO mice. Studies by Rubanyi *et al* (1997) suggested a role for ERα in regulating the basal release of NO. However, neither this study nor others have been designed to assess a role for ERα in the regulation of blood pressure in mice. In contrast, the characterisation of ERß in the cardiovascular system includes studies by Zhu *et al* who, using male mice, reported that ßERKO mice developed hypertension with age and that the loss of ERß lead to increased vascular tone (2002). In the previous chapter, *Chapter 4*, it was demonstrated that the loss of functional ERß in male mice led to enhanced contractility, similar to that reported by Zhu *et al*, and impaired endothelial function, as was concluded from the increase in sensitivity to the contractile agonist, PE. Impaired endothelial function was demonstrated as the basal release of NO, the stimulated release of NO and other endothelium- derived relaxant factors were reduced. However, the ability of vascular smooth muscle cells to relax in response to vasodilators was similar to that of WT mice. In our colony of animals,

altered vascular function due to loss of ERβ was not associated with alterations in blood pressure in male mice.

In *Chapter 3*, we demonstrated that ERβ was expressed in the mouse cardiovascular system. However, immunoreactive ERβ protein was also detected in the tissues of βERKO mice. Further investigation of the colony of βERKO mice established in our lab demonstrated that the ERβ protein expressed in our colony was unable to function as native WT ERβ protein. This was concluded from the observation that female βERKO mice presented with an ovarian phenotype similar to that described in the original targeting paper (Krege *et al*, 1998). The recent development of ERβ selective agonists and antagonists has allowed us to employ a different approach to studying the individual role of ERs as an alternative to ER knock out mice. Due to the detection of ERβ in our colony of βERKO mice we decided to take a more traditional approach to studying the role of ERβ in the female cardiovascular system and use the Organon ERβ selective antagonist.

Therefore, the aim of this study was to determine the effect of oestrogen status and the role of ERβ on blood pressure and vascular function using the novel ERβ selective antagonist.

5.2 Methods

5.2.1 Animals

Female C57Bl6 mice were purchased from Harlan UK and maintained as outlined in *Section 2.2.* To study the effect of oestrogen status and the role of ERβ on blood pressure and vascular function, animals aged 17- 19 weeks were assigned to one of three groups. The age of mice used in this study was selected due to the report by Zhu *et al* (2002) that differences in blood pressure between WT and βERKO mice were detected in male and female mice which were aged between 5- 6 months of age. In the first group, animals were allowed to continue their oestrous cycle, the stage of which was determine by vaginal smearing, as outlined in *Section 2.3.1.1.2.* Alternatively, animals underwent surgical bilateral ovariectomy and were supplemented with either oestrogen or placebo releasing pellets as outlined in *Section 2.3.1.1.1.* Animals were allowed a minimum recovery period of 7 days by which point body weight had returned that measured pre-operatively.

Study A

Animals were sacrificed 3 weeks post- surgery (20 -23weeks) and tissues harvested for *in- vitro* studies.

5.2.2 Radiotelemetric blood pressure measurement

Study B

Radiotelemetric devices were implanted in animals aged 18- 20 weeks, from each of the above experimental groups as outlined in *Section 2.3.1.2*, (sham ovx n= 5, ovx-E n = 5 and ovx+E n= 4). This enabled blood pressure (systolic, diastolic and mean pressure), heart rate and activity to be measured in conscious animals.

Following a 7 day recovery period, blood pressure, heart rate and activity were acquired continuously, rate of sampling was 10s every 15mins, *Figure 5.1.* To determine whether oestrogen influenced blood pressure, heart rate and activity, data

was acquired for animals in each of the groups; normally cycling females and those which had undergone ovx and administered placebo or supplemented with oestrogen. To determine the role of ERβ in mediating the effects of oestrogen on the parameters measured, the said ERβ selective antagonist (1mgkg -1, s.c.) was administered as instructed by Organon. A one hour window was scheduled between 10:30 and 11:30 to allow daily administration of the ERβ selective antagonist and to check the health status of each animal. As the vehicle for the ERβ selective antagonist was saline (Dulbecco's phosphate buffered saline, filter sterilised, Sigma- Aldrich, UK) vehicle controls were administered (~0.08ml, s.c.) for 3 consecutive days leading up to administration of ERβ selective antagonist. Upon completion of the vehicle controls, animals were treated with ERβ selective antagonist for 7 days prior to being euthanised and harvesting of tissues for *in- vitro* purposes, *Figure 5.1.*

At the onset of study B the appropriate vehicle control was unknown and therefore not every animal in each group was able to be administered vehicle. Once we were informed of the vehicle for ERβ selective antagonist without first doing a vehicle control. However, in those animals which had been administered vehicle, we found that the vehicle had an effect on blood pressure and therefore we had to compare ERβ selective antagonist within each oestrogen status group to that of the vehicle control. This meant that animals had to be excluded from each of these groups which had not received vehicle control. This reduced the number of animals in the experimental groups from 5 to 3 for those female mice which were normally cycling and from 4 to 3, those which had been ovx and supplemented with oestrogen.

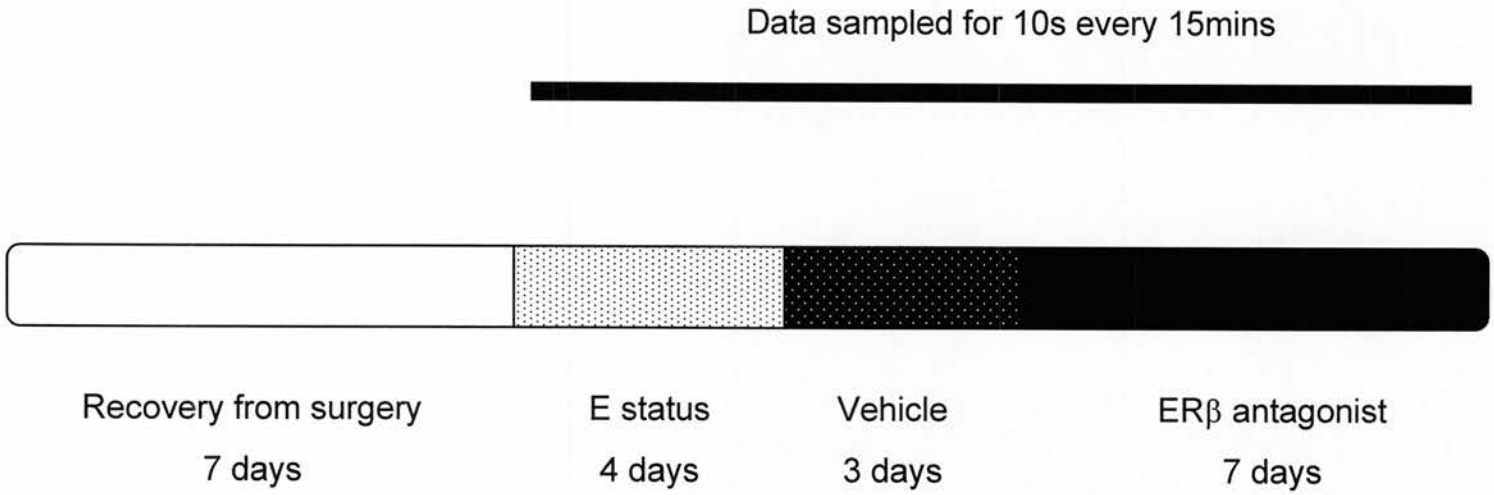

Figure 5.1 ***Timescale of radiotelemetry study***

Radiotelemetry devices were implanted into the mouse carotid artery and the body of the device implanted subcutaneously in female mice which had been assigned to Study B. To determine the effects of oestrogen status, data was acquired from female mice which were sham ovx, normally cycling (n=5), or had undergone ovx and administered either placebo (n=5) or supplemented with oestrogen (n=4). To determine the role of ERβ in mediating the effects of oestrogen on activity and haemodynamic parameters, the ERβ selective antagonist was administered chronically to mice which were sham ovx, normally cycling (n=3), or had undergone ovx and administered either placebo (n=3) or supplemented with oestrogen (n=3).

5.2.3 Assessment of vascular function

Mesenteric arteries were harvested from animals from both *Study A* and *Study B*. Therefore, the purpose and groups for the vascular study were; *Study A:* to assess the effect of oestrogen status on vascular function; normally cycling; n= 8, Ovx treated with placebo; n= 6 and Ovx supplemented with oestrogen; n= 7 and *Study B:* to determine the role of ERß in mediating the effects of oestrogen on vascular function; normally cycling; n= 8, normally cycling + ERβ selective antagonist; n= 7, Ovx treated with placebo; n= 6, ovx treated with placebo + ERβ selective antagonist; n= 6, Ovx and supplemented with oestrogen; n= 7 and Ovx supplemented with oestrogen + ERβ selective antagonist; n= 7.

Two rings of first order mesenteric arteries were obtained from the mesenteric vascular bed which was removed from each animal following exsanguination, mounted on the myograph and prepared for functional analysis as outlined in *Section 2.5*.

All of the vascular studies for this chapter were performed by Dr Pascual Medina.

5.2.3.1 Response to agonist induced contraction

Once the vessels were mounted and had undergone an equilibration period, the standard start protocol was carried out. The aim of the standard start protocol was not only to sensitise vessels to agonists but also to test the integrity of the vessel prior to the experimental protocol. Exclusion parameters were established such that vessels were only accepted for the study that had a contractile response to KCl greater than 500mg. In addition, to confirm the presence of endothelium, a relaxation response to ACh greater than 70% had to be obtained. Whilst establishing the technique in our lab, certain vessels obtained from mice in *Study A* and *Study B* did not meet the exclusion criteria of the protocol and therefore were not included in the analysis.

To determine whether oestrogen status was involved in regulating the contractile function of vascular smooth muscle cells, and the role of ERß in mediating these

effects, cumulative concentration response curves (CRC) to the α_1-adrenoceptor agonist, phenylephrine (PE, 10^{-9}- 10^{-6}M) was carried out on endothelium intact rings of first order mesenteric artery. Once the CRC was complete, PE was washed out and rings were allowed to re-equilibrate for 15 minutes before continuing with the experimental protocol.

5.2.3.2 Endothelium -dependent and -independent vasodilation

To investigate the response to vasodilating agents, the mesenteric artery, was pre-constricted by a submaximal concentration of PE (~EC_{80}). To assess endothelium-dependent relaxation and the stimulated release of nitric oxide from endothelial cells, a CRC to the cholinoceptor agonist acetylcholine (ACh, 10^{-10}- 10^{-5}M) was carried out on one of the two mesenteric rings obtained from each animal within each experimental group. To determine the ability of vascular smooth muscle cells to respond to NO, a CRC to the nitric oxide donor, sodium nitroprusside (SNP, 10^{-11}-10^{-6}M) was performed on the second of the 2 rings obtained from each animal. Once the CRC was complete, ACh or SNP, depending on the ring of mesenteric artery, was washed out and allowed to re- equilibrate for 15mins before continuing with the protocol.

5.2.3.3 Basal release of NO and prostanoids

One of the rings of mesenteric artery obtained from each animal was then incubated with the nitric oxide synthase inhibitor L-NAME (10^{-4}M, for 40min) which blocks the synthesis of NO. A CRC to PE was then constructed and the increased contractile response to PE, as compared to the CRC to PE in the absence of L-NAME, due to the block of NO synthesis by L-NAME was an indication of the basal level of NO activity.

The second ring of mesenteric artery obtained from the same animal as above, was incubated with indomethacin (10^{-5}M, for 40min) which inhibits the production of vasoactive prostanoids pathway by inhibiting the enzyme cyclooxygenase (COX). The CRC to PE was now constructed in the presence of indomethacin and the

response compared to that produced to PE in the absence of indomethacin. A variation in the extent of the decrease in the contractile response to PE by indomethacin would provide an indication of the basal levels of prostanoids released from the vessel.

5.3 Results

5.3.1.The effect of oestrogen status and the ERß selective antagonist on body and tissue weight.

Study A

The body weights of mice which had undergone sham ovx, and were normally cycling, or bilateral ovx with subsequent administration of placebo or oestrogen supplementation, were not found to be significantly different, *Table 5.1.* Tissues harvested from these animals were weighed and corrected for body weight. Female mice which had been supplemented with oestrogen following ovx had a significant increase in uterine weight when compared to both sham ovx and ovx mice administered placebo. Uterine weight of animals administered placebo was lower than that of normally cycling female mice, *Table 5.1.* As one of the parameters that the study was designed to assess was the effect of oestrogen on blood pressure, we also measured heart weight, in the event that any changes in blood pressure would have lead to hypertrophy of the heart. We found, however, that there was no difference in heart weight.

Study B

The body weight of animals which had been administered the ERβ antagonist was not found to be different to those animals which had not, nor was there any difference between animals of different oestrogen status receiving the ERβ antagonist, *Table 5.1.* Similar to that observed in animals which had not received the ERβ antagonist, placebo treated ovx animals which had received the ERβ antagonist, had a reduction in uterine weight compared to normally cycling female mice and those which had undergone ovx and supplemented with oestrogen. Similar to the increase in uterine weight observed in *Study A* between normally cycling females and those supplemented with oestrogen, in *Study B*, ovx mice supplemented with oestrogen and administered the ERβ antagonist had a higher uterine weight than normally cycling females administered the ERβ antagonist, *Table 5.1.* Treatment

with the ERβ antagonist did not affect uterine weight between animals of the same oestrogen status which had not received the ERβ antagonist. Once again we found that there was no significant difference in heart weight with oestrogen status in animals treated with or without the ERβ antagonist, or indeed between animals of the same oestrogen status treated with the ERβ antagonist and those which were not.

	Study A - ERβ antagonist			Study B + ERβ antagonist		
	Sham (n= 8)	Placebo (n= 6)	Oestrogen (n= 7)	Sham (n= 7)	Placebo (n= 6)	Oestrogen (n= 7)
Body weight (g)	22.7± 0.98	25.0± 0.88	24.4± 0.79	22.2± 0.98	24.0± 0.72	23.3± 0.80
Uterus (% BW)	0.40± 0.05	0.11± 0.03*	0.65± 0.04*$	0.3± 0.05	0.08± 0.01*	0.74± 0.09*$
Heart (% BW)	0.57± 0.04	0.52± 0.02	0.50± 0.02	0.53± 0.02	0.52± 0.01	0.54± 0.02

Table 5.1 ***Body and tissue weights for female mice***

*Female mice from Study A and Study B were sham ovariectomised or ovariectomised and administered either placebo or supplemented with oestrogen. Animals were weighed and the weight of harvested tissue were expressed as %age body weight. Values are expressed as mean± SEM and analysed using a 2- way ANOVA followed by a Bonferroni post- hoc test (oestrgen status x ERβ antagonist). *$p<0.05$ compared to sham ovariectomised animals and $^{\$}p<0.05$ compared to ovariectomised mice administered placebo.*

5.3.2. Regulation of blood pressure and haemodynamic parameters.

5.3.2.1. Influence of oestrogen on blood pressure, heart rate and activity

Study B

MABP was acquired by radiotelemetry in female mice of different oestrogen status; normally cycling, ovx administered placebo or ovx supplemented with oestrogen, *Figure 5.1*. MABP, over the course of the final 24hrs of the study period, was found to be significantly greater in mice ovx and supplemented with oestrogen than sham ovx female mice. MABP of those mice which had undergone ovx and received placebo was significantly lower when compared to sham ovx females, over a 24hr period, *Figure 5.2*.

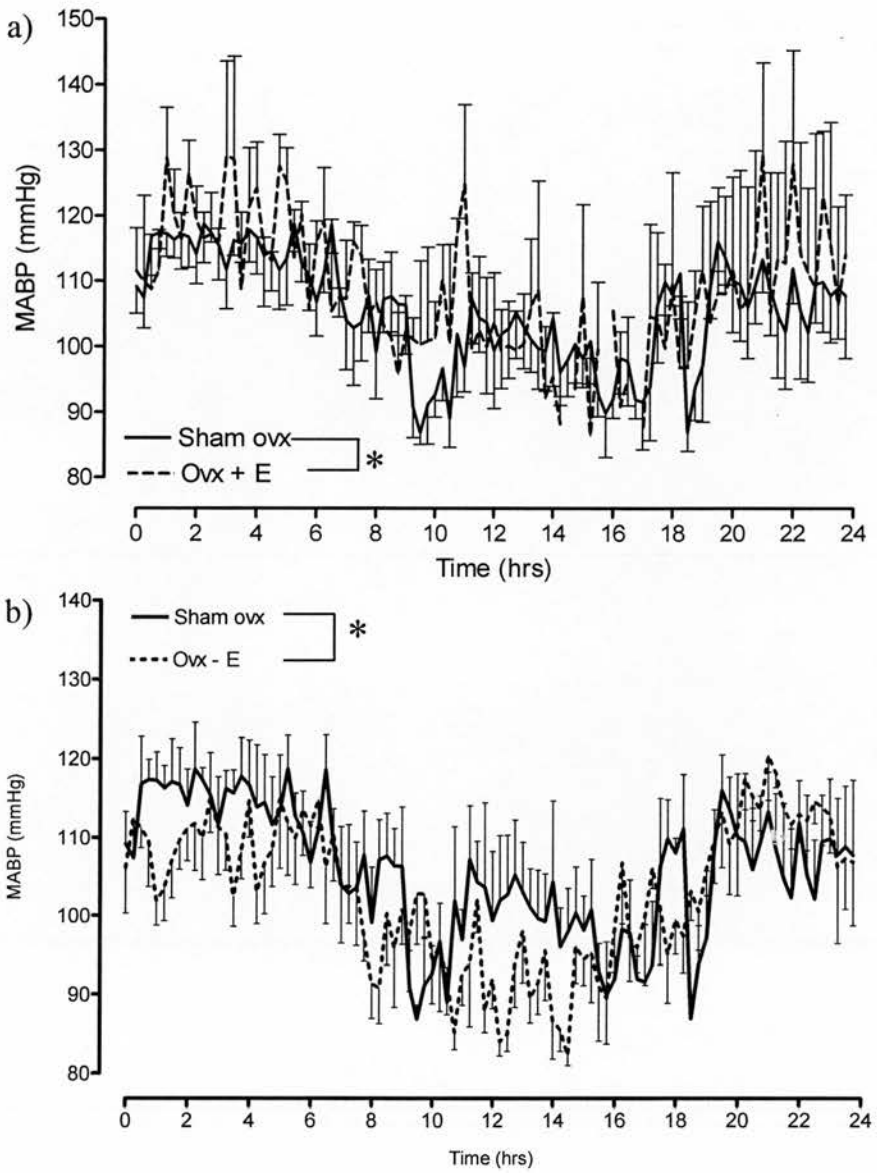

Figure 5.2 ***Mean arterial blood pressure acquired in female mice of different oestrogen status.***

*Mean arterial blood pressure (MABP) in sham ovariectomised (—,n= 5) or ovariectomised with either a) oestrogen (n= 4) or b) placebo (n= 5) supplementation (---). Data are expressed as the mean of data acquired every 15mins over the final 24hrs of each treatment group. Values are mean± SEM and analysed using a 2- way ANOVA (time x oestrogen status) *p<0.05 when compared to sham ovariectomised group*

Circadian variations in heart rate (HR), SBP, DBP, MABP and activity were determined by constructing 12hr rolling averages for animals in each of the oestrogen treatment groups studied. The 12hr periods were related to the 12hr light and dark cycles in the animal unit. The light period, termed "day" was from 0700 to 1900 and the dark cycle "night" from 1900 to 0700.

Heart rate

There was no significant difference in HR between sham ovx, ovx mice treated with placebo or oestrogen during either day or night, *Table 5.2*. However, HR was shown to increase in each of the three oestrogen treatments groups at night during which time the animals were also shown to be most active.

Blood pressure

SBP, DBP and MABP increased at night in each of the three oestrogen treatment groups. However, differences in blood pressure between mice of different oestrogen status during both day and night were also observed, *Table 5.2*.

MABP of placebo treated ovx animals was found to be lower than that of normally cycling female mice, *Figure 5.2*. Further analysis of blood pressure between these two groups demonstrated that MABP of placebo treated animals was less than that of sham ovx animals during the day but that there was no difference between these two groups at night, *Table 5.2*. As DBP was not found to be different between sham ovx and placebo treated mice the lower MABP in placebo treated mice may be associated with the reduction observed in SBP in this group. During the day, when MABP was lower than sham ovx, SBP was found to be reduced in placebo treated animals when compared to sham ovx mice, *Table 5.2*.

From *Figure 5.2*, it was observed that chronic oestrogen treatment of ovx female mice lead to an increase in MABP when compared to normally cycling female mice. MABP

was found to be greater than placebo treated animals during the day and both sham ovx and placebo treated animals at night, *Table 5.2*. This was not reflected in SBP during the time periods studied and therefore, the increase in MABP may be attributed to the differences in DBP following chronic oestrogen treatment, *Table 5.2*. DBP of ovx mice which received oestrogen was shown to be greater than that of sham ovx and placebo treated ovx mice during both day and night periods, *Table 5.2*.

Activity

The activity of mice in each of the three oestrogen groups studied was shown to increase at night when compared to the levels of activity during the day which is the animals natural inactive period, *Table 5.2*. However, oestrogen status appeared to affect the level of activity during both the inactive and active periods. Although the levels of activity were much less during the day than at night for each of the groups of mice, mice which had undergone ovx and administered either oestrogen or placebo were found to be less active than that of sham ovx animals. This reduction in activity in the oestrogen and placebo treated animals was also observed during the natural active period of mice, night, *Table 5.2*.

	DAY			NIGHT		
	Sham	Placebo	Oestrogen	Sham	Placebo	Oestrogen
HR (bpm)	574.1± 6.7	557.4± 5.1	564.8± 10.2	628.0± 5.9*	600.1± 5.2*	603.5± 9.8*
SBP (mmHg)	118.7± 0.9	112.6± 0.9$^{\$}$	116.6± 1.7	132.8± 0.9*	129.2± 0.9*	131.5± 1.6*
DBP (mmHg)	90.0± 1.0	87.0± 0.8	96.3± 1.3$^{\$\gamma}$	101.4± 0.9*	101.5± 0.8*	107.9± 1.6*$^{\$\gamma}$
MABP (mmHg)	99.6± 0.9	95.5± 0.8$^{\$}$	102.4± 1.5$^{\gamma}$	111.8± 0.8*	110.7± 0.7*	115.8± 1.6*$^{\$\gamma}$
Activity	8.8± 0.9	4.0± 0.4$^{\$}$	4.6± 0.5$^{\$}$	23.2± 1.5*	15.1± 0.9*$^{\$}$	12.9± 1.4*$^{\$}$

Table 5.2 ***Comparison of haemodynamic parameters and activity during day and night in female mice of different oestrogen status***

*Following radiotelemetry implantation, heart rate (HR), systolic (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MABP) and activity from the final 24hrs of each study period in female mice which had undergone sham ovariectomy (n=5), ovariectomy administered placebo (n=5) or supplemented with oestrogen (n=4) were analysed. Data were expressed as 12hr rolling averages from 0700 to 1900, for day measurements, and 1900 to 0700, for night measurements. Values were expressed as mean± SEM and analysed using a 2-way ANOVA (oestrogen status x time) followed by a Bonferroni post- hoc test. *$p<0.05$ relative to day measurements of animals of the same oestrogen status, $^{\$}p<0.05$ relative to sham ovx mice at the same time point and $^{\gamma}p<0.05$ relative to ovx mice administered placebo within the same time point.*

5.3.2.2. Influence of ERβ on the effects of oestrogen on blood pressure

Study B

To determine the influence of ERß on the observed effects of oestrogen on MABP, we analysed MABP following the administration of the ERβ selective antagonist ($1mgkg^{-1}$) over a 7 day period. The ERβ selective antagonist, was found to increase MABP recorded in normally cycling female mice relative to vehicle control during the final 24hr period of the last day of treatment, *Figure 5.3*. In contrast the ERβ selective antagonist, reduced MABP from vehicle in mice which had undergone ovx and been supplemented with oestrogen, as shown from data acquired over a 24hr period, *Figure 5.3*.

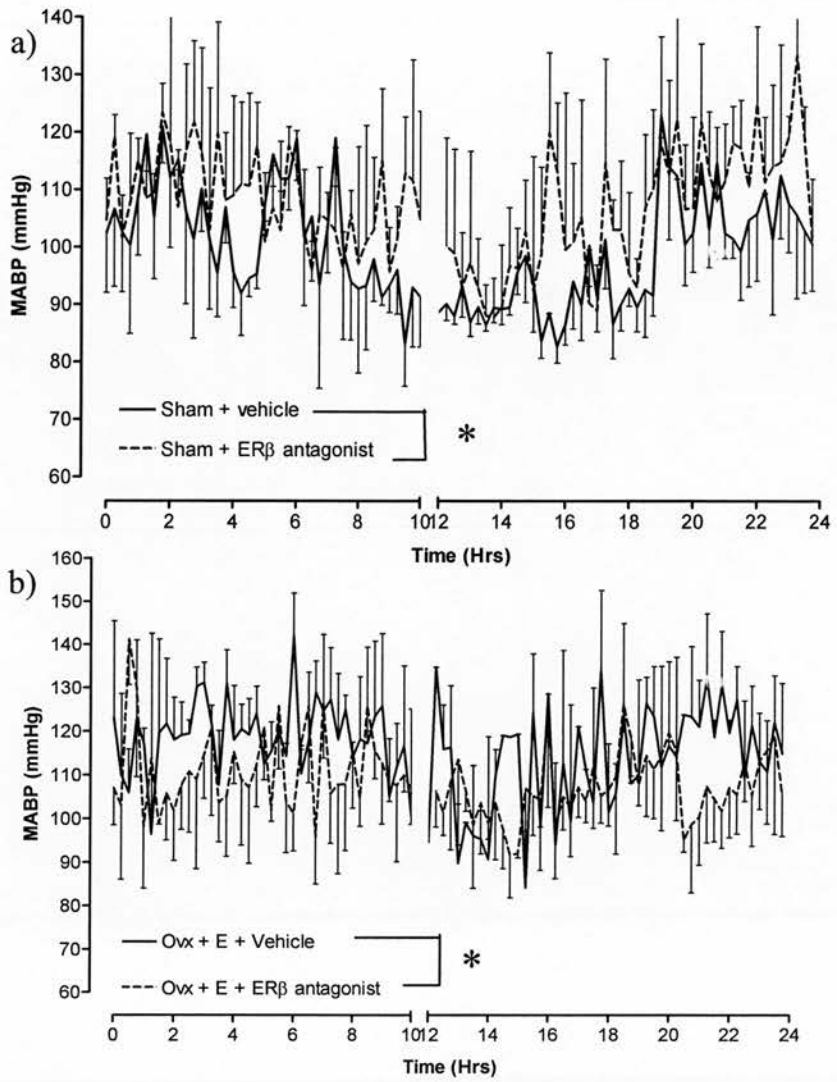

Figure 5.3 ***MABP in female mice following administration of the ERβ selective antagonist (1mgkg^{-1}).***

*Mean arterial blood pressure (MABP) recorded in female mice in the final 24hr period following 7day administration of the ERβ antagonist. Female mice which were either a) sham ovariectomised or b) ovariectomised and supplemented with oestrogen, were administered vehicle (—) prior to treatment with the ERβ antagonist (---). Data are expressed as the mean of data acquired every 15mins over the final 24hrs of each treatment period. Values are mean± SEM and analysed using a 2- way ANOVA (time x ERβ antagonist) *$p<0.05$ when compared to vehicle control group (n=3 in each group).*

5.3.2.2.1. Influence of ERβ on haemodynamic parameters in normally cycling female mice

Circadian variations in HR, SBP, DBP, MABP and activity, such that all of these parameters increased at night, were observed in normally cycling female mice whether during the administration period of vehicle or the ERβ antagonist in each animal.

Heart rate

Although HR increased at night compared with that measured during the day, HR in mice administered the ERβ antagonist was less than that during the vehicle treatment period of the same animals during both night and day, *Table 5.*3.

Blood pressure

SBP, DBP and MABP increased at night in normally cycling female mice during the periods of administration of vehicle or the ERβ antagonist, *Table 5.3.*

Administration of the ERβ antagonist to normally cycling female mice resulted in an increase in MABP when compared to vehicle control, *Figure 5.3.* The increase in MABP was shown to be elevated by the ERβ antagonist during both day and night relative to vehicle controls, *Table 5.*3. The increase in MABP during these periods may be attributed to the concurrent increase in DBP by ERβ antagonist but not to an effect on SBP as this was found to be unaffected by treatment with the antagonist in normally cycling female mice, *Table 5.3.*

Activity

The activity of normally cycling female mice was shown to increase at night when compared to the levels of activity during the day which is the animal's natural inactive period, *Table 5.3.* The level of activity in these mice was shown not to differ between the period of vehicle or ERβ antagonist administration, *Table 5.*3.

	Day		Night	
	Vehicle	ERβ antagonist	Vehicle	ERβ antagonist
HR (bpm)	603.9± 5.4	536.6± 7.0$^{\$}$	645.6± 6.7*	576.8± 7.5*$^{\$}$
SBP (mmHg)	113.0± 1.1	117.6± 1.7	127.1± 1.4*	132.0± 1.7*
DBP (mmHg)	83.6± 1.0	94.2± 2.0$^{\$}$	95.9± 1.2*	102.8± 1.6*$^{\$}$
MABP (mmHg)	93.4± 1.0	102.0± 1.9$^{\$}$	106.3± 1.3*	112.5± 1.6*$^{\$}$
Activity	5.7± 0.8	4.3± 0.6	24.4± 1.8*	24.9± 2.1*

Table 5.3 ***Comparison of haemodynamic parameters and activity during day and night in normally cycling female mice following administration of the ERβ selective antagonist.***

*Following radiotelemetry implantation, heart rate (HR), systolic (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MABP) and activity in female mice which had undergone sham ovariectomy (n=3) from the final 24hrs of the period of administration of vehicle control (3 days) and following the ERβ selective antagonist (7 days). Data were expressed as 12hr rolling averages from 0700 to 1900, for day measurements, and 1900 to 0700, for night measurements. Values were expressed as mean± SEM and analysed using a 2-way ANOVA (treatment x time) followed by a Bonferroni post- hoc test. *$p<0.05$ relative to day measurements of animals of the same treatment, $^{\$}p<0.05$ relative to vehicle control at the same time point.*

5.3.2.2.2. Influence of ERβ on haemodynamic parameters in ovx female mice treated with oestrogen

In contrast to the presence of circadian variations in ovx female mice treated with oestrogen, *Table 5.2*, during the period of vehicle and following that the administration of the ERβ antagonist, these animals showed no diurnal variation in HR, SBP, DBP or MABP *Table 5.4*.

Heart rate

HR was not different between day and night in female mice treated with oestrogen during the period of either vehicle or that of administration of the ERβ antagonist, *Table 5.*4.

Blood pressure

MABP, SBP and DBP were not different between day or night in female mice treated with oestrogen during the periods of administration of vehicle or the ERβ antagonist antagonist, *Table 5.4.*

Administration of the ERβ antagonist to oestrogen treated female mice resulted in a decrease in MABP when compared to vehicle control, *Figure 5.3b.* MABP was shown to be decreased by the antagonist during the night relative to the administration period of the vehicle control, *Table 5.*4. The decrease in MABP during this period may be attributed to the concurrent decrease in DBP by the ERβ antagonist but not to an effect on SBP as this was found to be unaffected by treatment with the antagonist in female mice treated with oestrogen, *Table 5.4.*

Activity

The activity of oestrogen treated female mice during the period of vehicle control administration was shown to increase at night when compared to the levels of activity during the day, *Table 5.4.* However, this pattern was not observed in the same animals during the period of administration of ERβ antagonist, as there was no difference in activity between night and day during this period, *Table 5.4.* This may be due to the increase in day time activity of mice treated with the antagonist relative to that of the vehicle control.

	Day		Night	
	Vehicle	ERβ antagonist	Vehicle	ERβ antagonist
HR (bpm)	531.4± 8.3	568.3± 8.4	569.7± 9.3	574.4± 7.7
SBP (mmHg)	130.8± 8.3	121.7± 1.6	131.7± 1.6	122.8± 1.7
DBP (mmHg)	105.6± 2.1	101.2± 1.5	112.4± 1.7	102.1± 1.6$^{\$}$
MABP (mmHg)	111.9± 3.1	107.2± 1.7	118.8± 1.6	109.0± 1.6$^{\$}$
Activity	4.3± 0.4	8.3± 0.8$^{\$}$	9.9± 0.8*	8.8± 0.9

Table 5.4 ***Comparison of haemodynamic parameters and activity during day and night in oestrogen treated ovx female mice following administration of the ERβ selective antagonist.***

*Following radiotelemetry implantation, heart rate (HR), systolic (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MABP) and activity in female mice which had undergone ovariectomy and supplemented with oestrogen (n=3) from the final 24hrs of the period of administration of vehicle control (3 days) and following the ERβ antagonist (7 days). Data were expressed as 12hr rolling averages from 0700 to 1900, for day measurements, and 1900 to 0700, for night measurements. Values were expressed as mean± SEM and analysed using a 2-way ANOVA (treatment x time) followed by a Bonferroni post- hoc test. *p<0.05 relative to day measurements of animals of the same treatment, $^{\$}p<0.05$ relative to vehicle control at the same time point.*

5.3.3 Analysis of vascular function

5.3.3.1 Influence of oestrogen on endothelial cell and vascular smooth muscle cell function

Study A

Phenylephrine

Cumulative addition of PE to first order mesenteric arteries from sham ovx, ovx supplemented with oestrogen and ovx treated with placebo female mice, led to contraction of the vascular smooth muscle, *Figure 5.4*. The response to 125mM KCl was similar between all groups (Sham ovx; 0.69± 0.05g, Ovx – E; 0.68± 0.08g, Ovx + E; 0.8±3 0.03g, 2- way ANOVA, (ERβ antagonist x oestrogen status) followed by a Bonferroni post- hoc test. Therefore the response to PE was expressed as a percentage of 125mM KCl. From *Figure 5.4a*, oestrogen supplemented female mice have an increased sensitivity to PE when compared to sham ovx mice as indicated by the leftward shift of the concentration response curve in oestrogen treated mice, *Table 5.5*. There was no difference in the contractile response to PE between sham ovx mice and those that were administered placebo, *Figure 5.4b*.

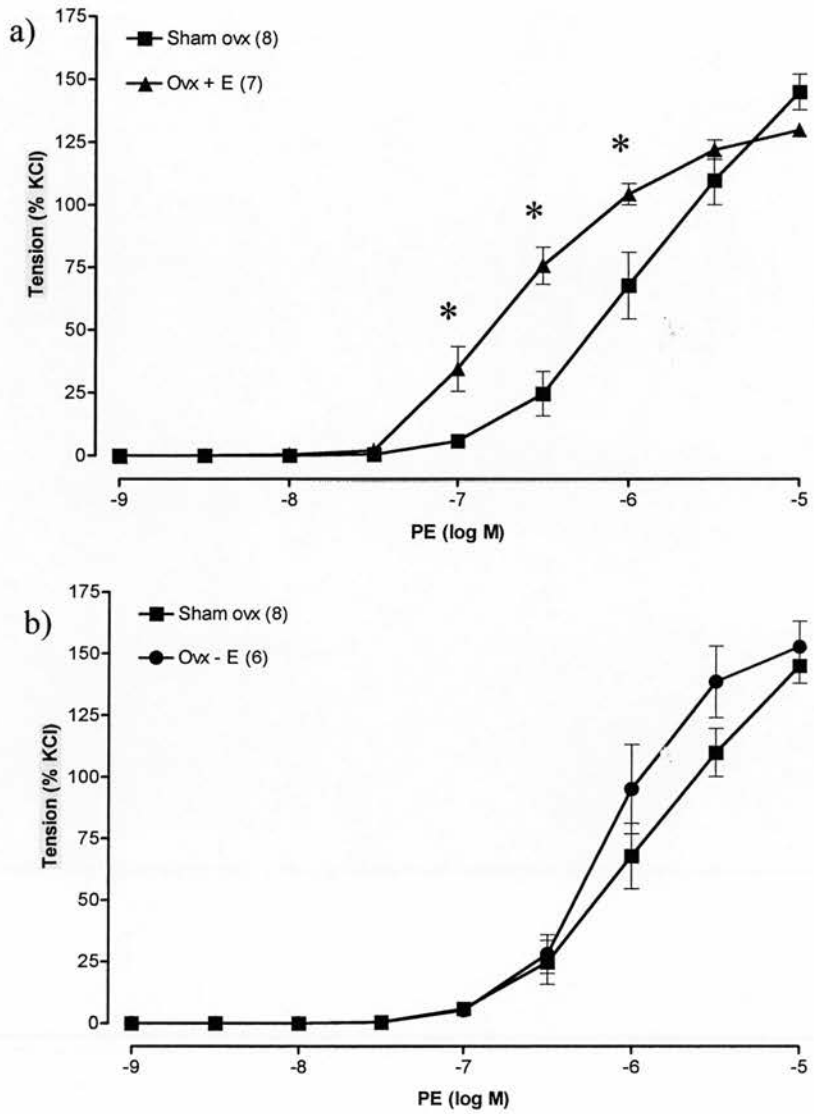

Figure 5.4 ***Response to PE in the vasculature of female mice***

*Cumulative concentration responses to PE in first order mesenteric artery rings with intact endothelium isolated from female, a) sham ovx (■) and ovx supplemented with oestrogen (π) and b) sham ovx and ovx mice treated with placebo (●)Values expressed as mean± SEM and analysed using a 2- way ANOVA followed by a Bonferroni post hoc test, *p<0.05*

	Study A - ERβ antagonist				Study B + ERβ antagonist			
	Sham (n=8)		Ovx + E (n=7)		Sham (n=7)		Ovx + E (n=7)	
	EC_{50} (M)	E_{max} (%KCl)	EC_{50} (M)	E_{max} (%KCl)	EC_{50} (M)	E_{max} (%KCl)	EC_{50} (M)	E_{max} (%KCl)
PE	$14.4x10^{-7}\pm 2.9x10^{-7}$	136.3± 10.0	$1.2x10^{-7}\pm 1.6x10^{-7}$*	126.7± 7.9	$11.9x10^{-7}\pm 2.8x10^{-7}$	152.0± 15.1	$11.0x10^{-7}\pm 3.0x10^{-7}$ $	140.3± 8.0
PE + L-NAME	$18.2x10^{-7}\pm 4.7x10^{-7}$	141.4± 8.0	$3.13x10^{-7}\pm 0.8x10^{-7}$*	131.2± 11.9	$17.9x10^{-7}\pm 7.1x10^{-7}$	153.0± 17.5	$13.4x10^{-7}\pm 3.5x10^{-7}$$	147.7± 12
PE + Indomethacin	$9.4x10^{-7}\pm 2.5x10^{-7}$	118.7± 15.7	$1.65x10^{-7}\pm 0.3x10^{-7}$*	119.0± 4.5	$7.1x10^{-7}\pm 1.2x10^{-7}$	138.5± 8.0	$8.5x10^{-7}\pm 1.1x10^{-7}$ $	144.0± 9.7

Table 5.5 ***Contractile responses to PE in the vasculature of female mice***

*The EC50 and Emax were calculated for the contractile effect of PE in the absence and presence of either L-NAME (10^{-4}M) or indomethacin (10^{-5}M) in isolated, endothelium intact first order mesenteric vessels. Vessels were isolated from female mice that were sham ovx or had undergone ovx and supplemented with oestrogen. Data are expressed as mean± SEM and analysed using a 2- way ANOVA (oestrogen status x ERβ antagonist), followed by a Bonferroni post- hoc test. $p<0.05$ *when compared to sham ovx, $ when compared to equivalent oestrogen status in Study A.*

L-NAME

Cumulative addition of PE in the presence of L-NAME (10^{-4}M) to first order mesenteric arteries from sham ovx, ovx supplemented with oestrogen and ovx treated with placebo female mice did not alter the contractile response to PE suggesting that in the vascular bed studied, NO is not the main mediator of basal tone. The increased sensitivity to PE and the similarity in the shape of the curve to PE between vessels from mice supplemented with oestrogen compared to sham ovx mice was still observed, *Figure 5.5a* and *Table 5.5*. This suggests that the increased sensitivity to PE in mice that were supplemented with oestrogen was not due to changes in NO availability.

The contractile response to PE in the presence of L-NAME was not shown to be different between sham ovx mice and those that were administered placebo, *Figure 5.5b.*

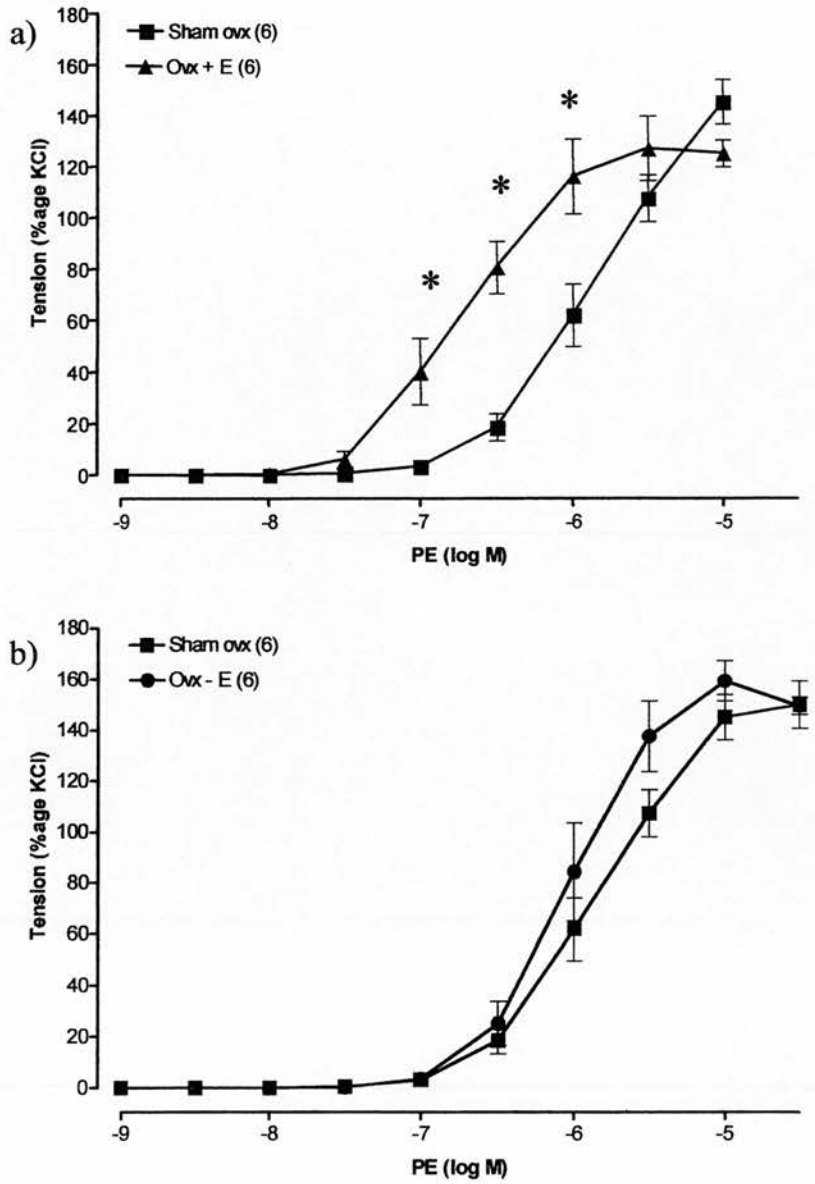

Figure 5.5 ***Response to PE in the presence of L-NAME in the vasculature of female mice***

*Cumulative concentration responses to PE in the presence of L-NAME (10^{-4}M), in first order mesenteric artery rings with intact endothelium. Vessels were isolated from female, a) sham ovx (■) and ovx supplemented with oestrogen (π) and b) sham ovx and ovx mice treated with placebo (●). Values expressed as mean± SEM and analysed using a 2- way ANOVA followed by a Bonferroni post hoc test, *p<0.05*

Indomethacin

Cumulative addition of PE in the presence of indomethacin (10^{-5}M) did not alter the contractile response to PE in first order mesenteric vessels from sham ovx, *Figure 5.6a*, or ovx mice which were administered placebo. However, vessels from ovx mice supplemented with oestrogen incubated with indomethacin resulted in a leftward shift of the PE CRC, *Figure 5.6b*. Although the EC_{50} and the E_{max} for the response to PE in the presence of indomethacin were not different to that of PE alone, *Table 5.5*, *Figure 5.6b* suggests that chronic oestrogen treatment increased the release of vasodilatory prostanoids.

The increased sensitivity to PE in ovx mice supplemented with oestrogen when compared to sham ovx mice, *Figure 5.4a*, remained in the presence of indomethacin, *Figure 5.7a.* Although indomethacin resulted in a slight leftward shift of the contractile response to PE in ovx mice supplemented with oestrogen, *Figure 5.*6b, it was not found to further enhance the difference in contractile response between sham and oestrogen supplemented mice (dose ratio: sham ovx; 1.93± 0.41, ovx + E; 0.9± 0.25, unpaired t-test). This suggests that despite an increase in vasodilatory prostanoids from the endothelium, chronic oestrogen supplementation still resulted in increased contraction to PE.

There was no significant difference in the contractile response to PE in the presence of indomethacin in vessels from sham ovx or ovx mice treated with placebo, *Figure 5.*7b.

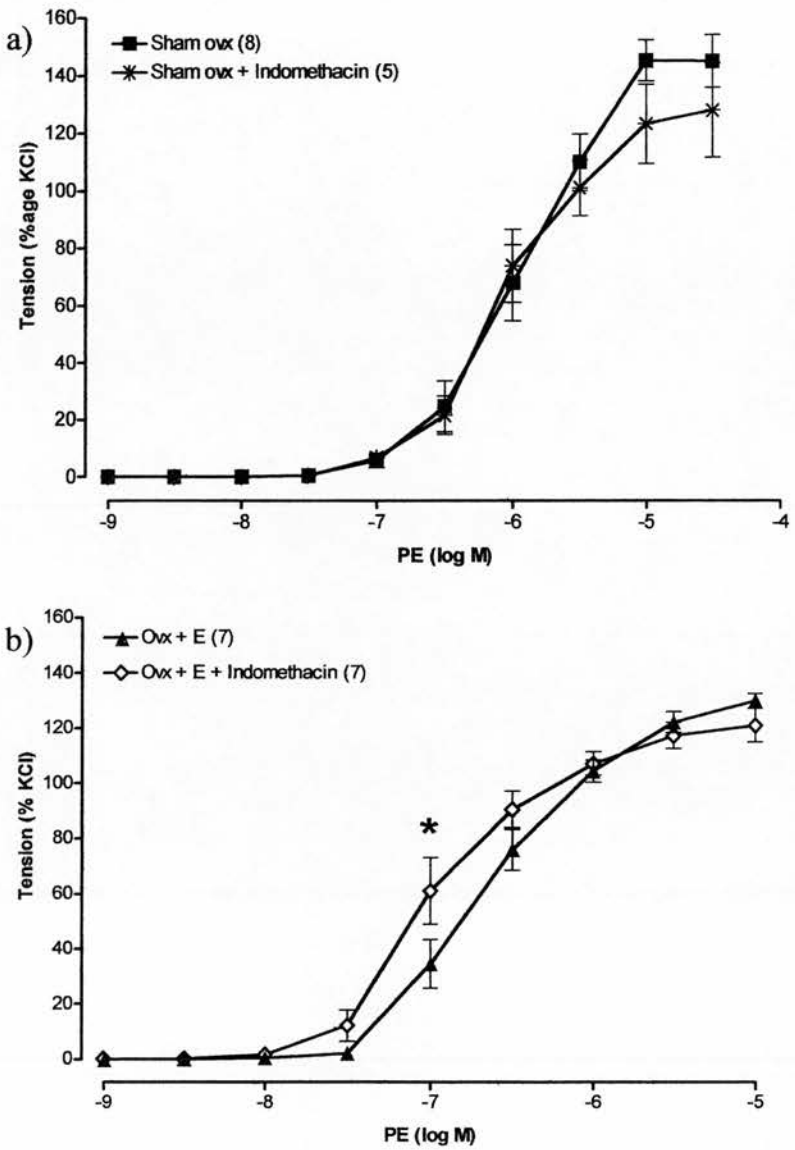

Figure 5.6 ***Response to PE in the presence of Indomethacin in the vasculature of female mice***

*Cumulative concentration responses to PE in the presence of indomethacin (10^{-5}M) in first order mesenteric artery rings with intact endothelium. Vessels were isolated from female a)sham ovx and b) ovx mice supplemented with oestrogen. Values expressed as mean± SEM and analysed using a 2- way ANOVA followed by a Bonferroni post hoc test, *p<0.05*

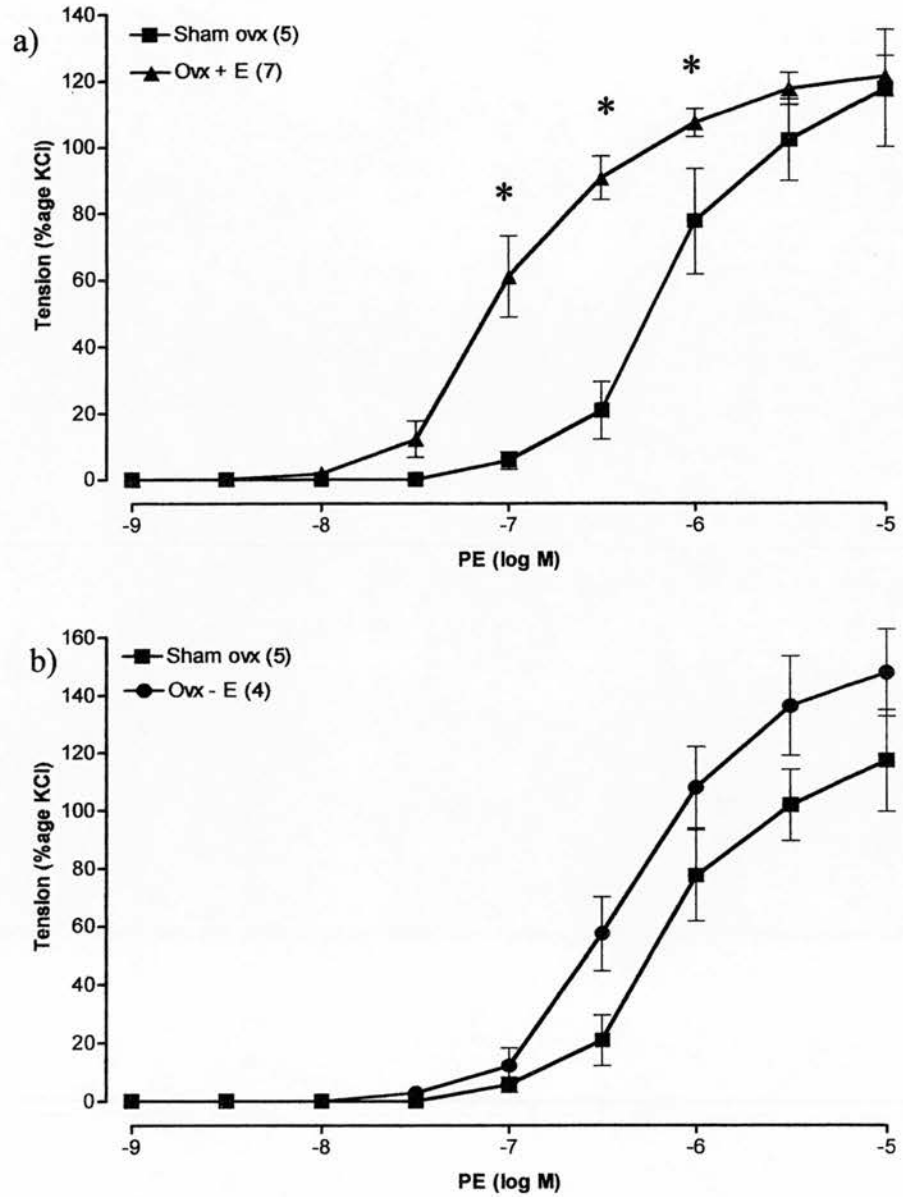

Figure 5.7 ***Response to PE in the presence of Indomethacin in the vasculature of female mice***

*Cumulative concentration responses to PE in the presence of indomethacin ($10^{-5}M$) in first order mesenteric artery rings with intact endothelium. Vessels were isolated from female a)sham ovx (■) and ovx supplemented with oestrogen (π) and b) sham ovx and ovx mice treated with placebo (●). Values expressed as mean± SEM and analysed using a 2- way ANOVA followed by a Bonferroni post hoc test, *$p<0.05$*

ACh and SNP

To assess endothelium- dependent and -independent relaxation in vessels from sham ovx and mice ovx and supplemented with oestrogen, mesenteric arteries were preconstricted with PE (~EC_{80}) prior to constructing a CRC to ACh in one of the rings and SNP in the second of the rings obtained from the animal. The response to PE, to preconstrict the vessels, was similar between each of the groups (sham ovx; 0.63± 0.1g, ovx treated with placebo; 0.78± 0.02g and ovx supplemented with oestrogen; 0.65± 0.08g, 2-way ANOVA, (ERβ antagonist x oestrogen status)) and therefore relaxation was expressed as a percentage of the contraction produced in response to PE. Mesenteric arteries from ovx mice supplemented with oestrogen had a decreased sensitivity to ACh, *Figure 5.8a* and SNP, *Figure 5.8b* when compared to the relaxation response of vessels from sham ovx mice. The relaxation response to ACh and SNP was not different between sham ovx and ovx mice administered placebo, *Figure 5.9a* and *Figure 5.9b*.

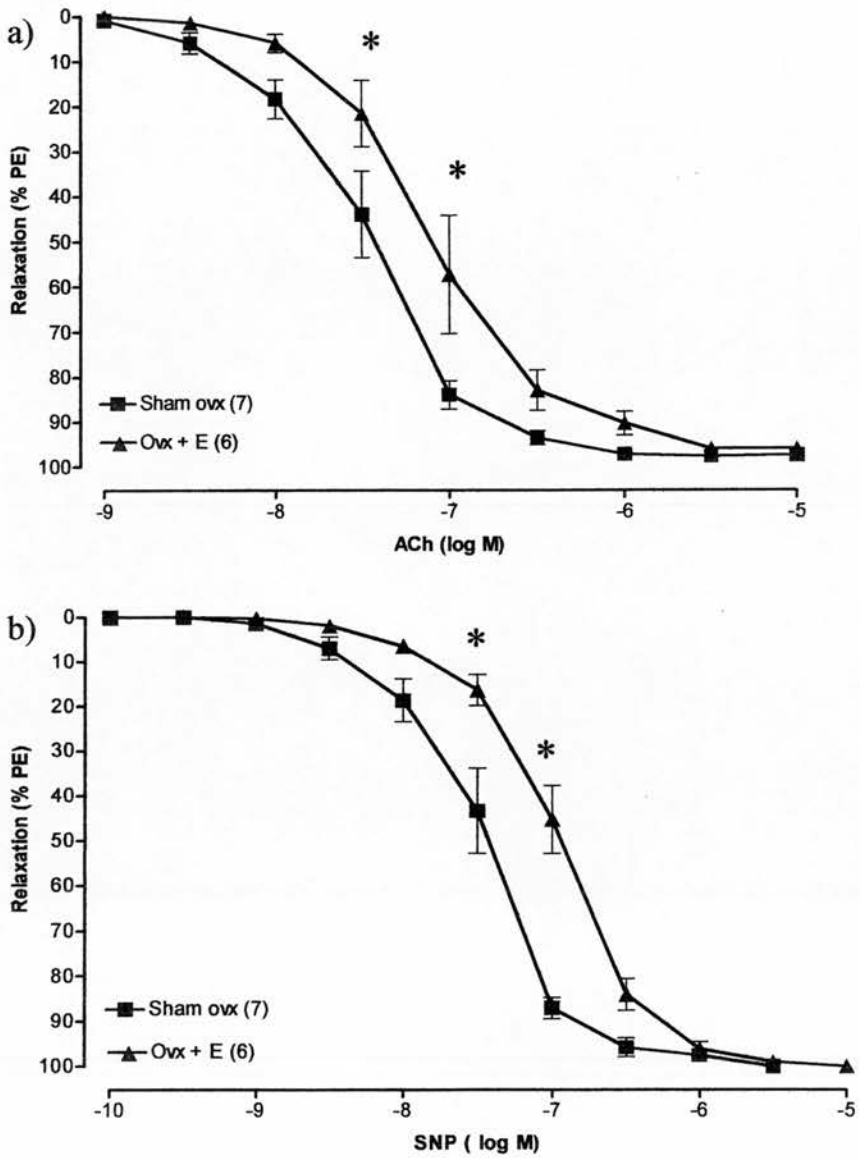

Figure 5.8 ***Response to ACh and SNP in the vasculature of female mice***

*Cumulative concentration response to a) ACh and b) SNP in first order mesenteric artery rings with intact endothelium in vessels, preconstricted with PE (~EC_{80}), isolated from sham ovx (■) and ovx + oestrogen supplementation (π). Values expressed as mean± SEM and analysed using a 2- way ANOVA followed by a Bonferroni post hoc test, *$p<0.05$*

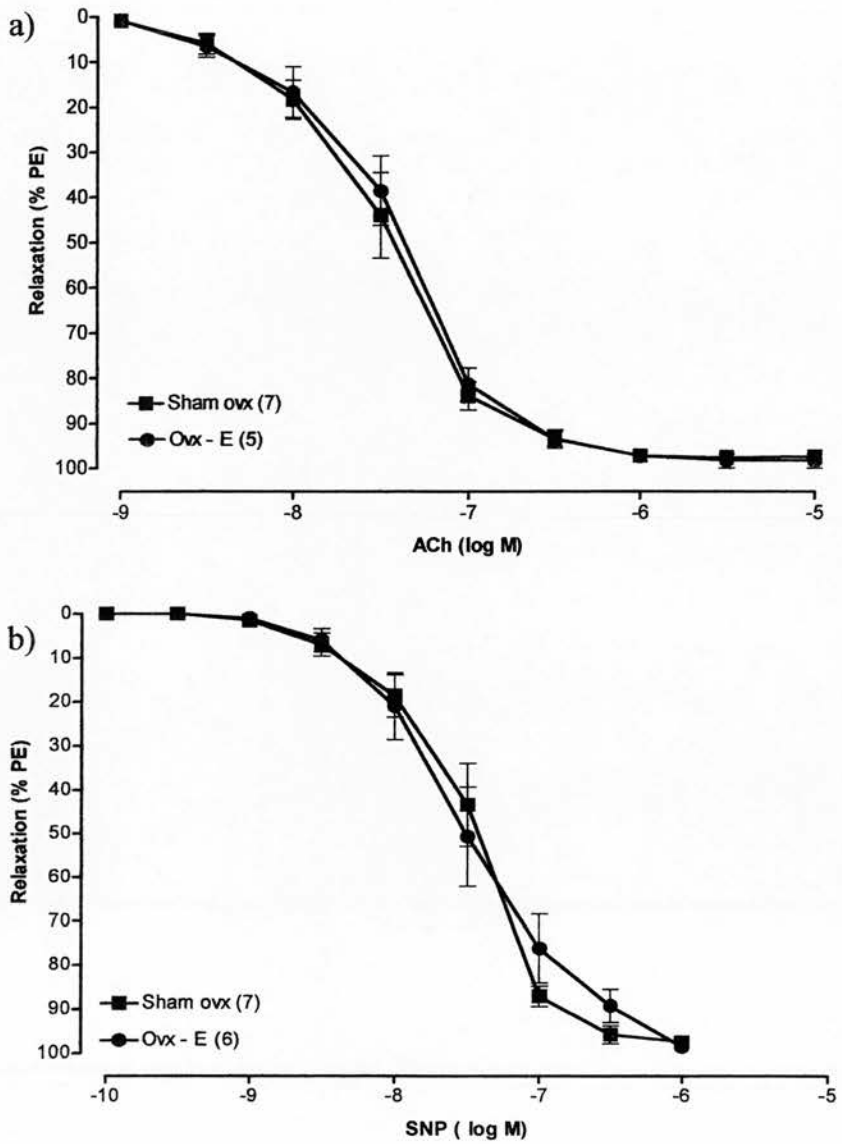

Figure 5.9 ***Response to ACh and SNP in the vasculature of female mice***

Cumulative concentration response to a) ACh and b) SNP in first order mesenteric artery rings with intact endothelium in vessels, preconstricted with PE (~EC_{80}), isolated from sham ovx (■) and sham ovx and ovx mice treated with placebo (●). Values expressed as mean± SEM and analysed using a 2- way ANOVA.

5.3.3.2 Influence of ERß in mediating the effects of oestrogen on the female mouse vasculature

The influence of ERß in mediating the effects of oestrogen on the female mouse vasculature, was determined by studying vessels from animals treated with the ERβ selective antagonist for a period of 7 days, *Study B*. Vessels were harvested from female mice that were normally cycling or ovx and supplemented with either oestrogen or placebo. Data was compared with the vessels used in *Study A* that did not receive the ERß antagonist.

5.3.3.2.1 Normally cycling female mice

PE CRC

The contractile response to PE in first order mesenteric arteries from sham ovx female mice following treatment with the ERβ antagonist was not found to be different when compared to sham ovx mice which had not been treated with the antagonist, *Figure 5.10* and *Table 5.5*.

PE CRC + L-NAME

The contractile response to PE in vessels incubated with L-NAME was not different between vessels from sham ovx mice treated with the ERβ antagonist and those which were not, *Figure 5.11a,* and *Table 5.5*. This indicates that treatment of mice with the ERβ antagonist, did not alter NO bioavailability in mice whose oestrogen levels were normally cycling.

PE CRC + Indomethacin

The contractile response to PE in the presence of indomethacin was not different between sham ovx mice that had been treated with the ERβ antagonist and those which were not *Figure 5.11b* and *Table 5.5*. This suggests that the antagonist did not alter the release of prostanoids in normally cycling female mice.

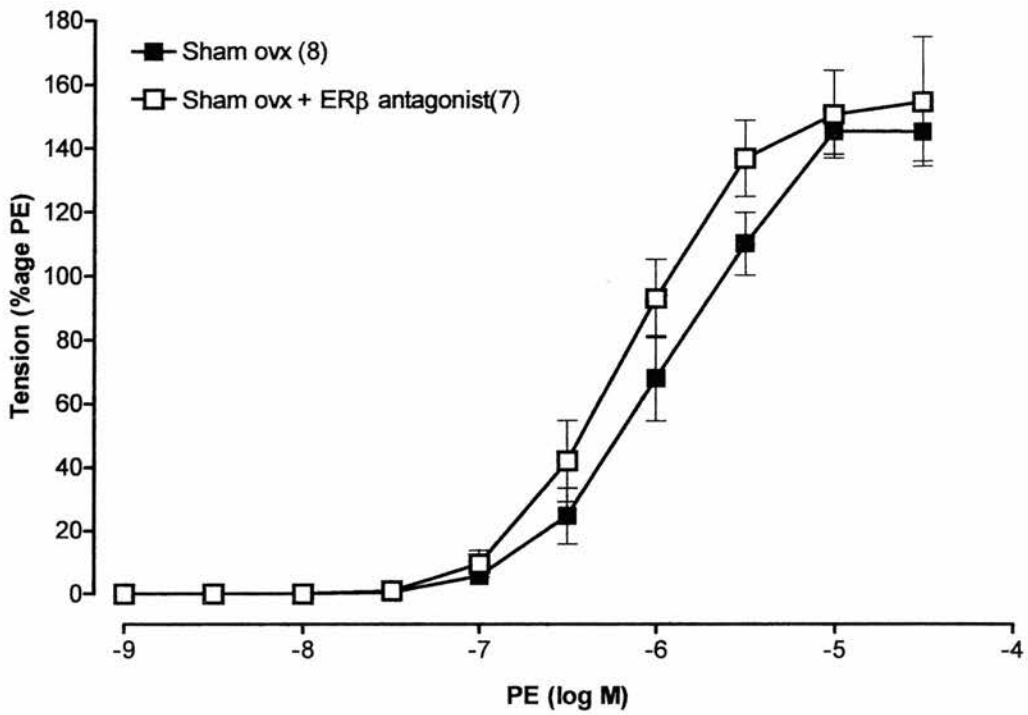

Figure 5.10 ***Response to PE in the presence of the ERβ selective antagonist in sham ovx female mice***

Cumulative concentration response to PE in first order mesenteric artery rings with intact endothelium isolated from sham ovx (■) and sham ovx + ERβ selective antagonist (□). Values expressed as mean± SEM and analysed using a 2- way ANOVA (conc x ERβ antagonist).

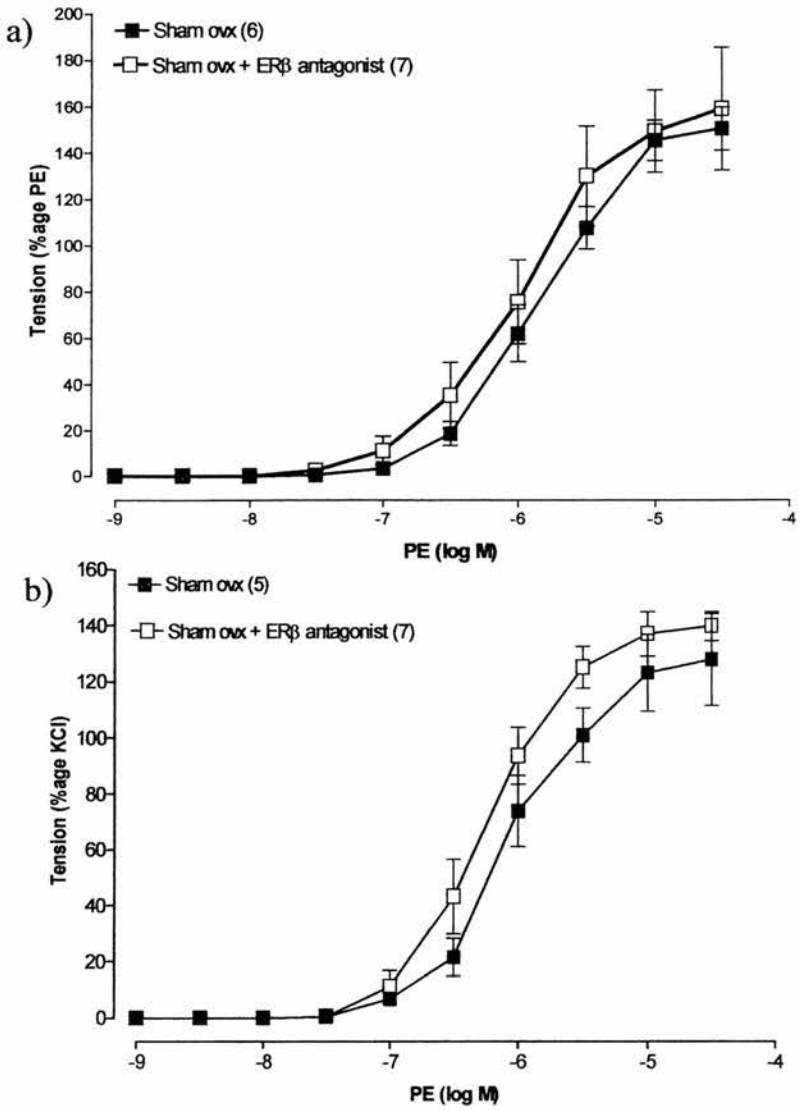

Figure 5.11 ***Response to PE in the presence of L-NAME and Indomethacin in the vasculature of female mice treated with ERβ antagonist***

Cumulative concentration responses to PE in the presence of a) L-NAME (10^{-4}M) and b) Indomethacin (10^{-5}M) in first order mesenteric artery rings with intact endothelium. Vessels were isolated from female, sham ovx (■) and female sham ovx + ERβ antagonist (□). Values expressed as mean± SEM and analysed using a 2- way ANOVA (concentration x ERβ antagonist)

Endothelial –dependent and –independent relaxation

The sensitivity to endothelium dependent relaxation in response to ACh in mesenteric arteries preconstricted with PE (EC_{80}) significantly increased in vessels from sham ovx animals treated with the ERβ antagonist when compared to vessels from sham ovx, *Figure 5.12a.* However, treatment with the antagonist did not alter the ability of vascular smooth muscle cells to relax as the relaxation response to the endothelium independent vasodilator SNP was not found to be different to that of sham ovx animals, *Figure 5.12b.*

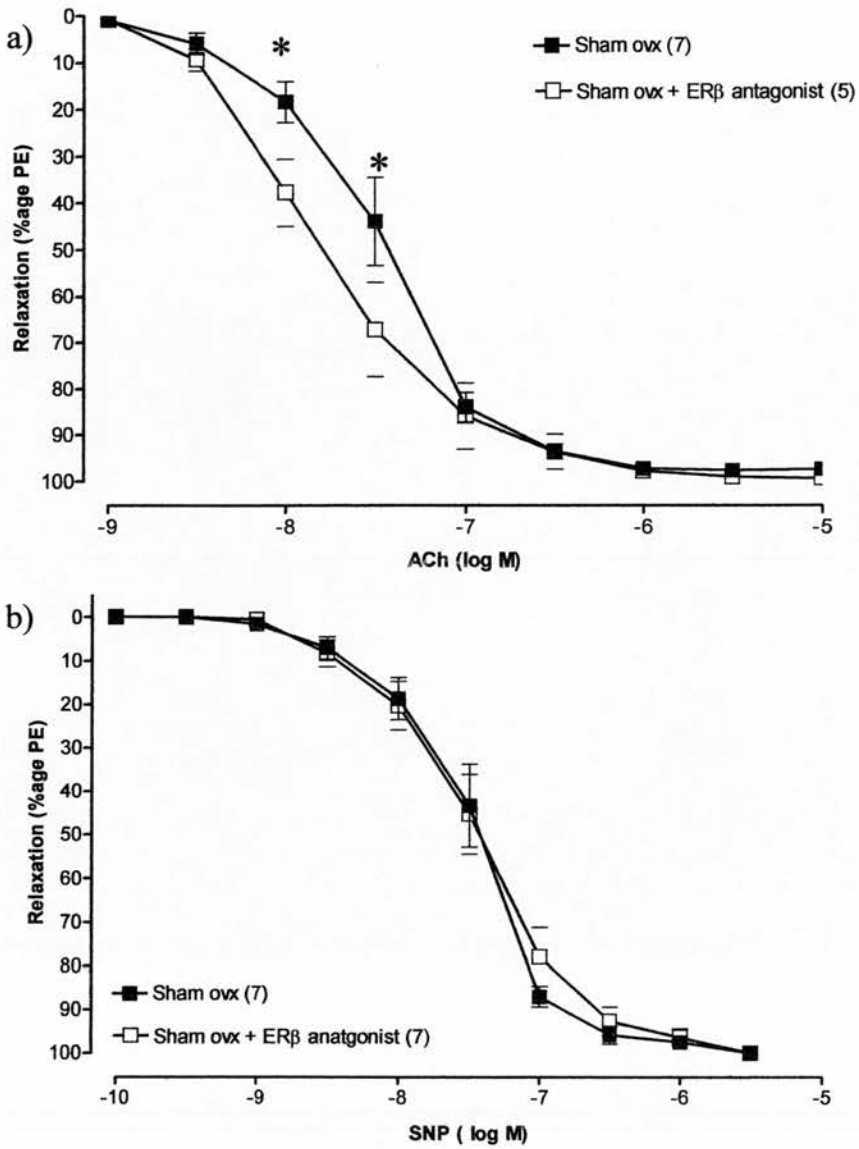

Figure 5.12 ***Response to ACh and SNP in the vasculature of normally cycling female mice treated with the ERβ selective antagonist***

*Cumulative concentration response to a) ACh and b) SNP in first order mesenteric artery rings with intact endothelium in vessels, preconstricted with PE (~EC_{80}), isolated from sham ovx (■) and sham ovx treated with the ERβ selective antagonist (□). Values expressed as mean ± SEM and analysed using a 2-way ANOVA (concentration x ERβ antagonist) followed by a Bonferroni post hoc-test *p<0.05*

PE CRC

The CRC to PE in mesenteric arteries from female mice that were ovariectomised and supplemented with oestrogen, was shifted to the right in animals treated with the ERβ antagonist when compared to those which were not, *Figure 5.13a* and *Table 5.5*. This suggests that, in animals supplemented with oestrogen, the ERβ antagonist inhibits the effect of oestrogen on enhancing vessel sensitivity to PE.

PE CRC in the presence of L-NAME

The contractile response to PE in the presence of L-NAME was shifted to the right in vessels obtained from mice that had been treated with the ERβ antagonist, *Figure 5.13b* and *Table 5.5*. The sensitivity of vessels from mice supplemented with oestrogen to PE in both the absence and in the presence of L-NAME was reduced in vessels obtained from animals which had been treated with the ERβ antagonist. Therefore, this suggests that the inhibitory effect of the ERβ antagonist on the increased sensitivity to PE as a result of chronic oestrogen treatment cannot be attributed to differences in the bioavailability of basal NO.

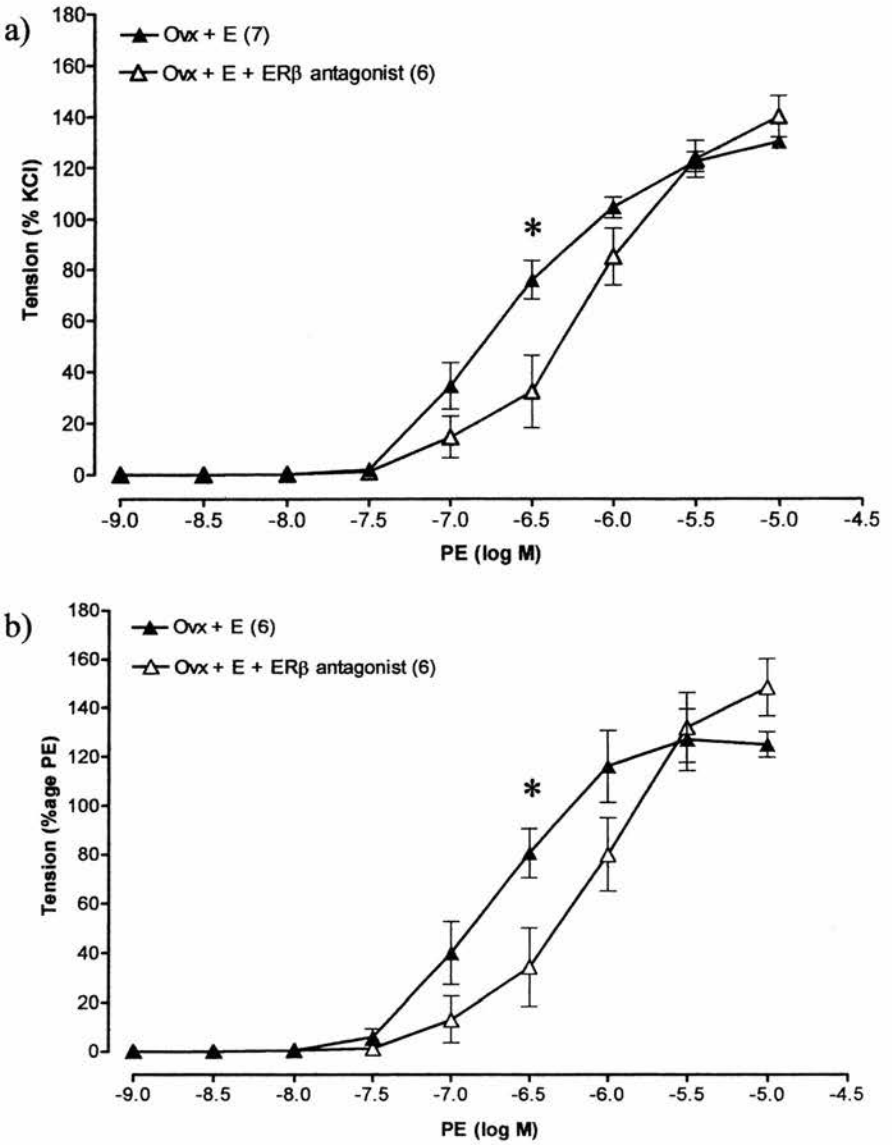

Figure 5.13 ***Response to PE in the absence and presence of L-NAME in the vasculature of ovariectomised female mice supplemented with oestrogen and treated with the ERβ selective anatgonist***

*Cumulative concentration responses to PE in a) the absence and b) the presence of L-NAME (10^{-4}M) in mesenteric artery rings with intact endothelium. Vessels were isolated from female mice which had undergone ovx and supplemented with oestrogen (π) and those which were also treated with the ERβ selective anatgonist (Δ,). Values expressed as mean± SEM and analysed using a 2- way ANOVA followed by a Bonferroni post hoc test, *p<0.05 (concentration x ERβ antagonist).*

PE CRC in the presence of Indomethacin

Vessels obtained from mice which had received chronic oestrogen supplementation caused a slight shift to the left of the PE CRC in the presence of indomethacin, *Figure 5.6*. In contrast, in vessels from mice supplemented with oestrogen which had been treated with the ERβ selective antagonist, the contractile response to PE was unaltered by indomethacin, *Figure 5.14a*. Therefore, this suggests that the ERβ antagonist blocks the release of vasodilating prostanoids from the endothelium induced by chronic oestrogen supplementation.

In the presence of indomethacin, the ERβ antagonist reduced the sensitivity to PE in mice supplemented with oestrogen when compared to vessels from mice which were not treated with the antagonist, *Figure 5.14b*. However, the dose ratio to PE was found to be greater in the presence of indomethacin of oestrogen supplemented mice treated with the antagonist when compared to those which were not (dose ratio: ovx + E; 0.9± 0.25, Ovx + E + ERβ antagonist; 1.37± 0.23, unpaired t- test).

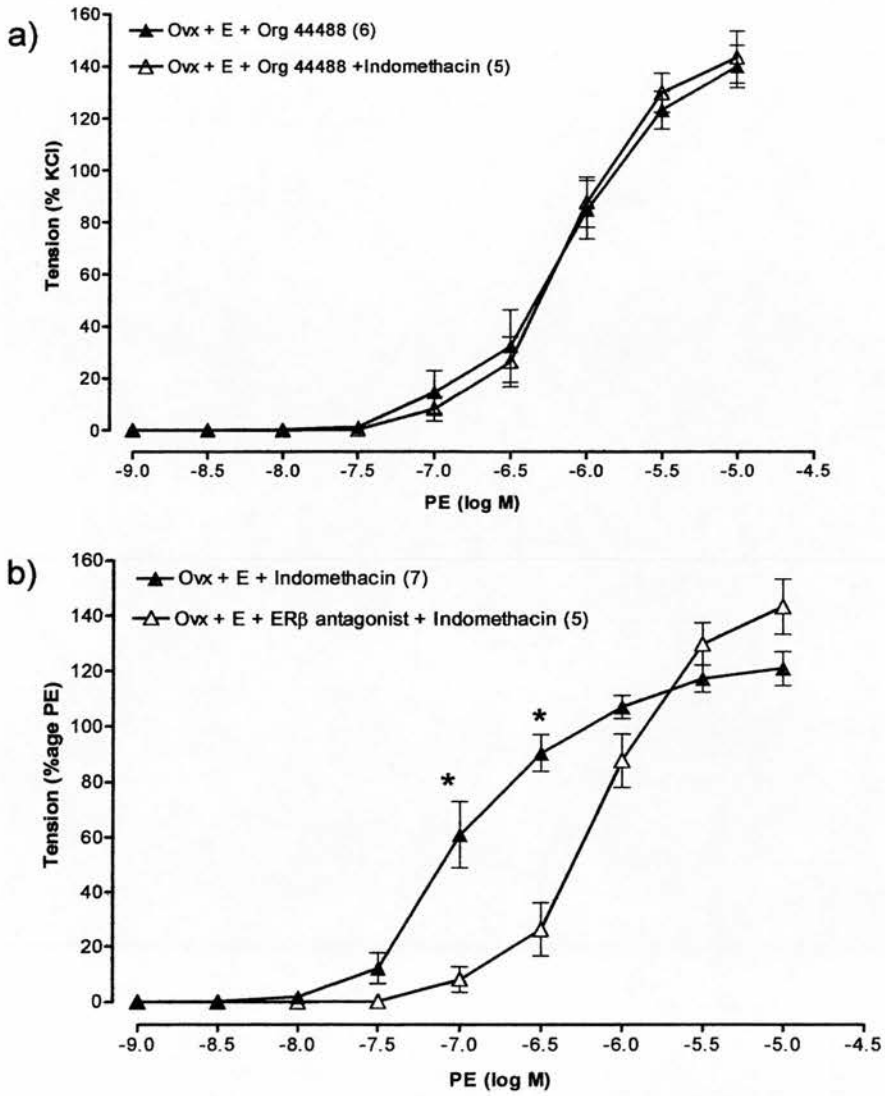

Figure 5.14 ***Response to PE in the presence of Indomethacin in the vasculature of ovariectomised female mice supplemented with oestrogen and treated with the ERβ selective antagonist***

*Cumulative concentration responses to PE in first order mesenteric artery rings with intact endothelium obtained from ovx female mice supplemented with oestrogen. Vessels were isolated from a) mice treated with the ERβ selective antagonist and response to PE in the absence (π) or presence (ρ) of indomethacin and b) response to PE in the presence of indomethacin in vessels from mice treated with vehicle (π) or the ERβ selective antagonist (ρ). Values were expressed as mean SEM and analysed using a 2- way ANOVA followed by a Bonferroni post hoc test, * $p<0.05$ (concentration x ERβ antagonist).*

Endothelium –dependent and –independent vasorelaxation

The endothelium –dependent and -independent relaxation to ACh and SNP respectively was different between ovariectomised mice supplemented with oestrogen and those which had also received the ERβ selective antagonist, *Figure 5.15.* Treatment with the ERβ antagonist, increased the sensitivity of vessels from female mice which were supplemented with oestrogen to the endothelial dependent relaxation response to ACh as indicated by a leftward shift of the concentration response curve, *Figure 5.15a.* The increase in sensitivity to the NO donor drug, SNP following treatment with the ERβ selective antagonist implies that the antagonist, in mice supplemented with oestrogen enhances vascular smooth muscle cell relaxation, *Figure 5.15b.* This suggests that the increase vasorelaxation produced in vessels from mice supplemented with oestrogen and treated with the ERβ antagonist is due to enhanced sensitivity of vascular smooth muscle cells to NO.

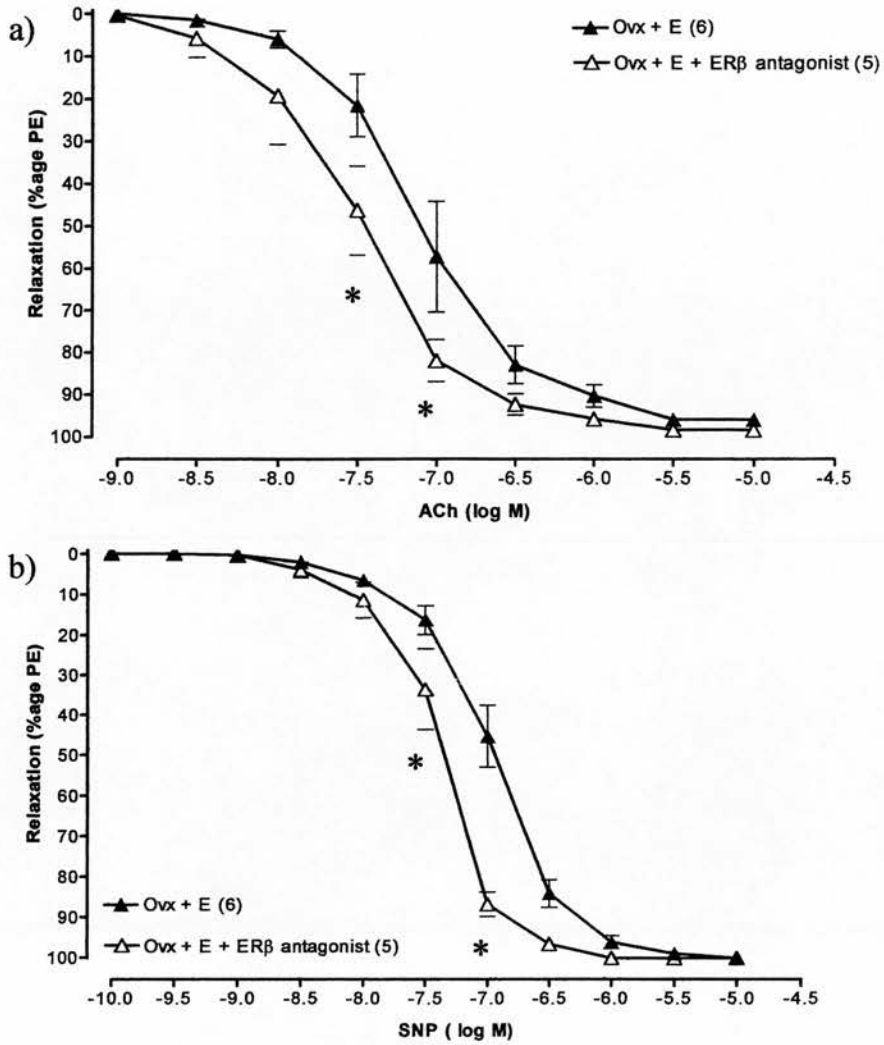

Figure 5.15 ***Relaxation response to ACh and SNP in the vasculature of ovariectomised female mice supplemented with oestrogen and treated with the ERβ selective antagonist***

Cumulative concentration response to a) ACh and b) SNP in first order mesenteric artery rings with intact endothelium in vessels preconstricted with PE (~EC_{80}), isolated from ovx female mice supplemented with oestrogen (π) and those which were treated with the ERβ selective antagonist (Δ). Values are expressed as mean± SEM and analysed using a 2- way ANOVA (concentration x ERβ antagonist), followed by a Bonferroni post hoc test $*p<0.05$.

5.3.3.2.3 Ovariectomised female mice treated with placebo

PE CRC

The contractile response to PE in first order mesenteric arteries from sham ovx female mice following treatment with the ERβ selective antagonist was not found to be different when compared to ovx mice administered placebo which had not been treated with the ERβ antagonist, *Figure 5.16.*

PE CRC + L-NAME

The contractile response to PE in the presence of L-NAME was not different between mice which had undergone ovx, administered placebo and treated with the ERβ antagonist and those which were not *Figure 5.17a.* This indicates that treatment with the antagonist did not alter NO bioavailability in ovariectomised mice which had been treated with placebo

PE CRC + Indomethacin

The contractile response to PE in the presence of indomethacin was not different between mice which had undergone ovx and treated with placebo and treated with the ERβ antagonist and those which were not *Figure 5.17b.* This suggests that the antagonist did not alter the release of prostanoids in ovx female mice treated with placebo.

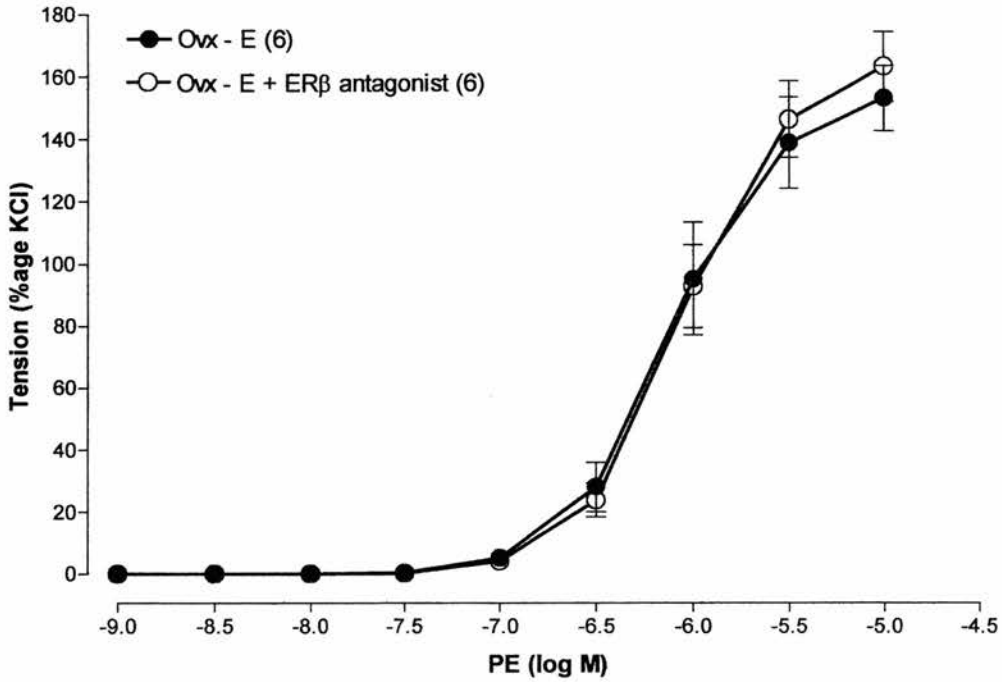

Figure 5.16 ***Response to PE in the presence of the ERβ selective antagonist in Ovx female mice treated with placebo***

Cumulative concentration response to PE in first order mesenteric artery rings with intact endothelium isolated from ovx mice treated with placebo (●) and ovx treated with placebo and administered the ERβ selective antagonist (O). Values expressed as mean± SEM and analysed using a 2- way ANOVA (conc x ERβ antagonist)

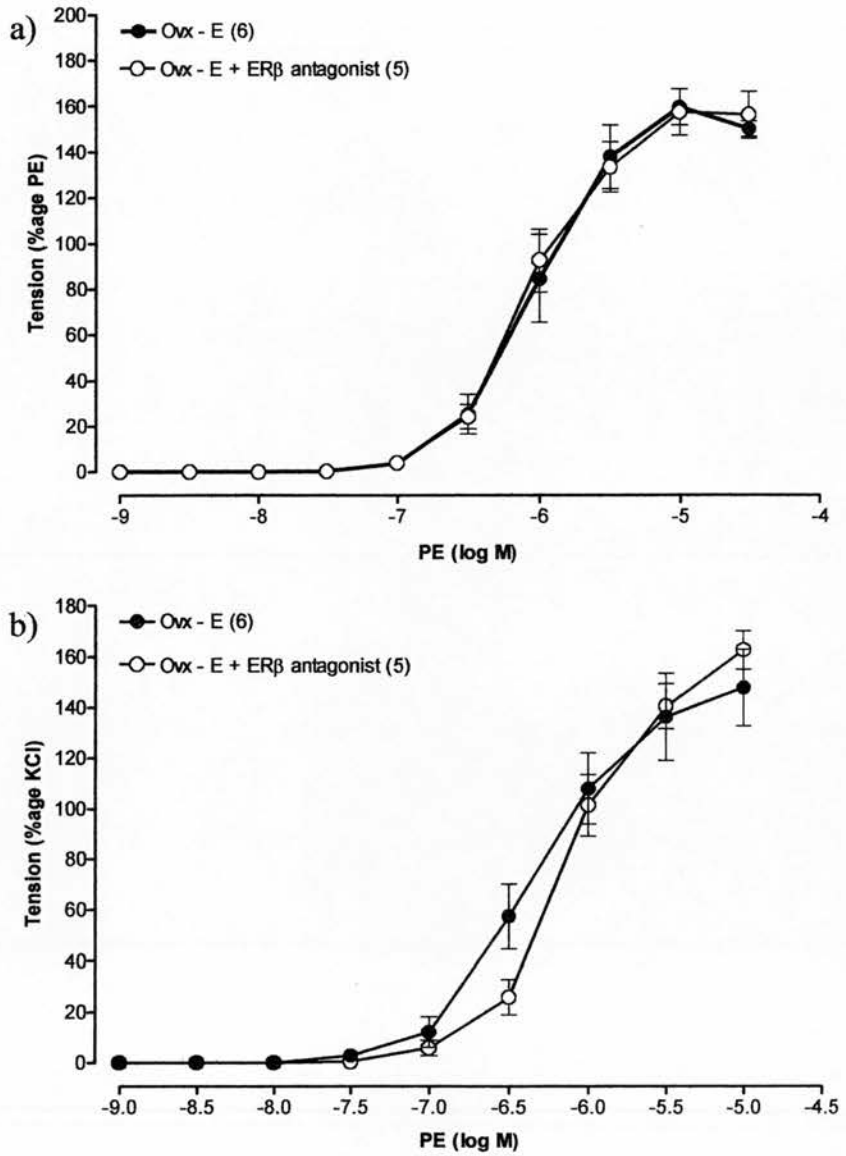

Figure 5.17 ***Response to PE in the presence of L-NAME and Indomethacin in the vasculature of ovariectomised female mice administered placebo and treated with the ERβ selective antagonist***

Cumulative concentration responses to PE in the presence of a) L-NAME (10^{-4}M) and b) Indomethacin (10^{-5}M), in first order mesenteric artery rings with intact endothelium. Vessels were isolated from female, ovx and treated with placebo (●) and ovx treated with placebo + ERβ antagonist (○). Values expressed as mean± SEM and analysed using a 2- way ANOVA (concentration x ERβ antagonist)

Endothelial –dependent and –independent relaxation

The sensitivity to endothelium dependent relaxation produced to ACh in mesenteric arteries preconstricted with PE (EC_{80}) was not different between vessels from ovx animals administered placebo and treated with the ERβ selective antagonist when compared to vessels from those which were not, *Figure 5.18a.* In addition, treatment with the antagonist did not alter the relaxant ability of vascular smooth muscle cells as the relaxation response to the endothelium independent vasodilator SNP was not found to be different to that of placebo treated mice, *Figure 5.18b.*

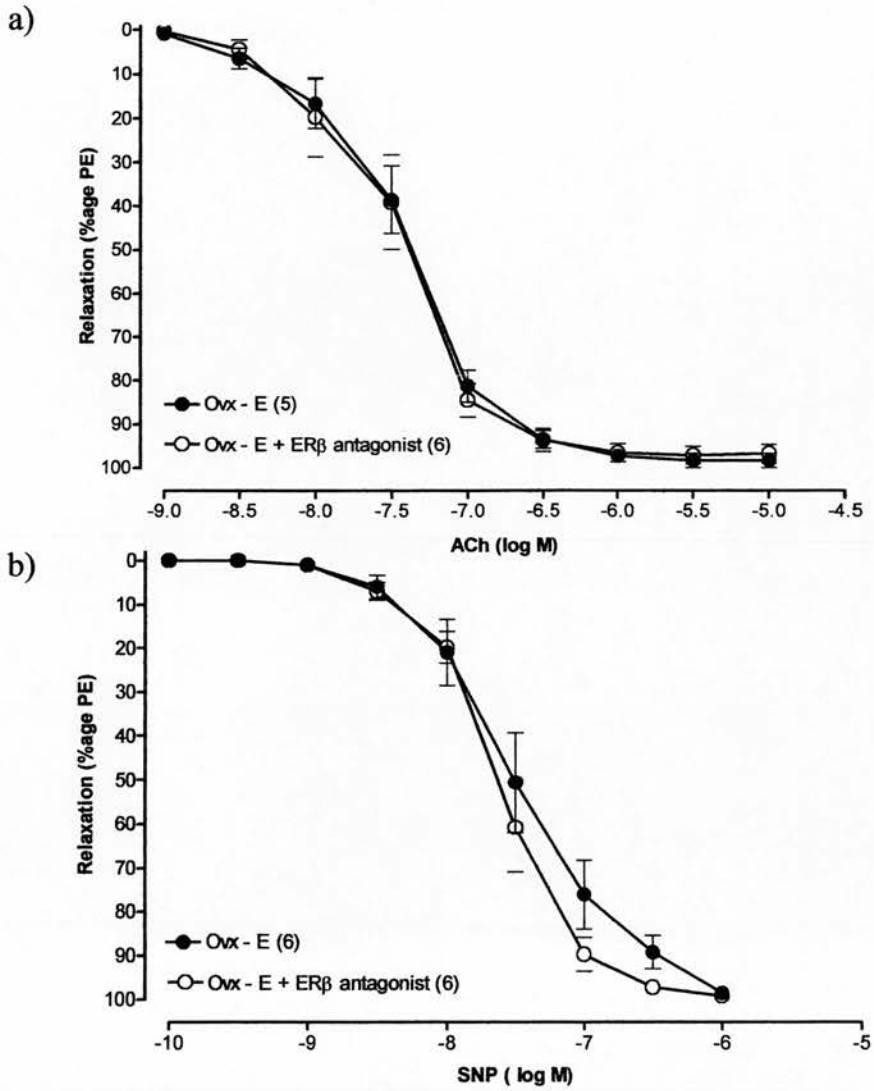

Figure 5.18 ***Response to ACh and SNP in the vasculature of female ovariectomised mice administered placebo and treated with the ERβ selective antagonist***

Cumulative concentration response to a) ACh and b) SNP in first order mesenteric artery rings with intact endothelium in vessels, preconstricted with PE (~EC_{80}), isolated from ovx female mice treated with placebo (●) and those treated with the selective ERβ antagonist (○). Values expressed as mean ± SEM and analysed using a 2-way ANOVA (concentration x ERβ antagonist).

5.4 Discussion

The novelty of this study was, that for the first time an ERß selective antagonist, a proprietary Organon compound, was used as an alternative method to that of knock-out animals to study the role of ERß. This study was designed to determine the effect of oestrogen, and the role of ERß in mediating those effects, on blood pressure and vascular function.

To study the effect of oestrogen on blood pressure and the vasculature, C57Bl6 mice were assigned to one of three groups; sham ovx, ovx treated with placebo or supplemented with oestrogen. During the oestrous cycle oestrogen concentration fluctuates and reaches its peak during proestrous. In this study, the individual cycle of each animal was known for the days leading up to and including the experiment end point. To control for the cycling levels of oestrogen in female mice, the ovaries, the main site of oestrogen synthesis, were surgically removed and animals either treated with placebo or supplemented with oestrogen. In this study we demonstrated that placebo treated animals were an appropriate model of oestrogen deprivation as there was a marked decrease in uterine weight due to atrophy of the uterus. The concentration of oestrogen, $50pgml^{-1}$, that was chosen to supplement animals that had been ovx was within physiological levels and equal to the concentration of oestrogen in the mouse during proestrous (Jabonlea- Shariff *et al*, 1999). Administration of either placebo or oestrogen to ovariectomised animals was via implantation of subcutaneous pellets as described in *section 2.3.1.1.1.* Proestrous is the stage during which ovarian follicular development occurs and is prior to oestrous, the period of ovulation. The animals in this study which were normally cycling were known to be either dioestrous, i.e. entering into proestrous, or met- oestrous i.e. coming out of oestrous. Therefore, it is likely that their average circulating oestrogen levels were lower when compared to those which were ovx and supplemented with oestrogen, although the concentration of oestrogen was not measured. The limitation of this study was the low number of animals in each group studied using telemetry, particularly sham ovx mice. By increasing the sample size, we would better observe the overall effect of endogenous oestrogen in each stage of the oestrous cycle.

Blood pressure was measured in animals from each group following implantation of radiotelemetry devices. Recently, the development of such devices for implantation in small rodents has enabled physiological measurements to be taken from conscious and free moving mice without introducing stress artefacts. It also provided a more favourable alternative to techniques such as the tail- cuff method and exteriorised catheters, which are limited as although the tail cuff method is non- invasive, it is known to be affected by environmental and physiological factors (Bunag, 1983, Krege *et al*, 1995). The use of exteriorised catheters introduces stress artefacts in the measurements taken as animals have to be tethered (Mattson, 1998). Much research has been committed to the development for implantation into small rodents such that devices can be implanted into mice as small as 17g (Butz and Davisson, 2001). In our study we inserted the catheter into the left carotid artery and placed the transmitter subcutaneously on the left flank. This was chosen as not only was catheterisation of the carotid a less invasive operation procedure, it was also reported in literature to have a greater degree of success than catheterisation of the aorta, > 90% and < 20% respectively which can result in the occlusion of blood flow to the hindlimbs (Carlson and Wyss, 2000). In addition, collateral flow from the right carotid artery is sufficient to perfuse the left side of the head and face, due to the Circle of Willis. Catheterisation of the left carotid artery was chosen to avoid aortic disruption which may have occurred if the right carotid was selected.

In this study we demonstrated that female mice which had undergone ovx and been supplemented with oestrogen had a small but significant increase in MABP when compared to that of sham ovx animals or those which were ovx and administered placebo. SBP was not found to be elevated in oestrogen treated animals and therefore the increase in MABP was attributed to the concomitant increase in DBP. During the day, oestrogen treatment lead to an increase of 6.3mmHg and 9.3mmHg in DBP, compared to sham ovx and placebo treated animals respectively. Similar to that observed during the day, at night DBP was elevated in animals treated with oestrogen when compared to sham ovx and placebo treated animals (an increase of

7.5mmHg and 6.4mmHg compared to sham ovx and placebo treated animals respectively).

This study also suggests that the presence of both endogenous and exogenous oestrogen increased blood pressure as mice administered oestrogen and those which were sham ovx had a higher mean arterial blood pressure, as measured over a 24hr period, than those which were ovx and administered placebo. The lower MABP of placebo treated mice was shown to occur during the day and can be attributed to the reduction in SBP in the placebo treated group (reduction of 6.1mmHg compared to normally cycling females). A decrease in systolic blood pressure such as that observed in placebo treated animals may be attributed to an increase in aortic distensibility. A study by Tatchum-Talom *et al*, would support such a finding as they demonstrated in rats that oestrogen deprivation lead to a reduction in aortic stiffness when compared to oestrogen treated ovx rats, which were found to have increased aortic stiffness associated with increase pulse pressure and left ventricular hypertrophy (2002). However, whether oestrogen deprivation would lead to a reduction in aortic stiffness in mice would require further investigation by pulse wave velocity and determining the elastin:collagen ratio.

The finding in this study that exogenous oestrogen resulted in a small but significant increase in MABP when compared to that of mice which were normally cycling may be surprising. Early clinical observations and animal studies report that oestrogen either had no effect on blood pressure, as with longitudinal clinical studies (Pearson *et al* 1997), or that blood pressure was lowered following oestrogen supplementation, in cross over clinical studies (Anastos *et al* 1991) and in a knockout mouse model of menopause associated hypertension (Javeshghani *et al*, 2003). However, it has been known for some time that one of the harmful effects of combined oral contraceptives (COCs) was an increase in blood pressure (Nichols *et al*, 1993, Qifang *et al*, 1994). The long- term use of COCs can increase a woman's risk two- fold of developing hypertension (Chasan- Taber *et al* 1996).

The mechanisms by which oestrogen may increase blood pressure are poorly understood. It is possible that the increase in blood pressure observed following oestrogen treatment is mediated by direct effects of oestrogen on the vasculature, or indirectly by modulating the renin angiotensin system (RAS), renal function or sympathetic nervous system.

Contradictory findings have been reported regarding the effects of oestrogen on the RAS as some studies have shown that oestrogen attenuates the RAS and reduces blood pressure (Nickenig *et* al, 1998, Roesch *et al*, 2000). In contrast, an oestrogen response element has been described in the angiotensinogen gene promoter (Feldmer *et al*, 1991) and oestrogen administration has been reported to stimulate the hepatic synthesis of angiotensinogen as observed in post-menopausal women receiving ERT (Schunkert *et al*, 1997). This has also been shown in animal models, where both physiological and pharmacological concentrations of oestrogen increased circulating levels of angiotensinogen in female rats (Stavreus- Evers *et al*, 2001) and that it was directly proportional to oestrogen concentration as angiotensinogen levels were high during oestrous and low during metoestrous in the rat brain (Greenland & Sernia, 2004).

In addition to activation of the renin- angiotensinogen, oestrogen may also increase blood pressure by altering renal function. A study of acute sodium loading in young pre-menopausal women reported that following suppression of endogenous oestrogen and progesterone, administration of exogenous oestrogen and progesterone increased distal tubule sodium reabsorption and reduced sodium excretion (Stachenfeld *et* al, 2004). As progesterone could not produce these effects independently, they were attributed to oestrogen. A reduction in sodium excretion and an increase in water retention contribute to an increase in blood pressure (Hall *et al*, 1980). However, studies have reported that oestrogen is not associated with an increased blood pressure response to salt. A study by Pechere- Bertschi *et* al, in which young women using oral contraceptives, who were salt resistant, responded to low salt intake by increasing sodium reabsorption and decreasing reabsorption in response to sodium loading (2003). This was similar to non- oral contraceptive users

who were salt resistant regardless of the stage of the menstrual cycle (Pechere-Bertschi *et al*, 2000). However, this group reported that although systemic blood pressure was not affected, oestrogen influenced the renal haemodynamic response to salt (Pechere- Bertschi *et al*, 2003). Women taking oral contraceptives had an increased glomerular filtration rate and an increase in proximal reabsorption of sodium which they suggested may be due to an increase in RAS. Therefore human models suggest that the increase of blood pressure in response to oestrogen observed in our study is more likely to involve increased activity of the RAS than an altered pressure-natriuresis response. Further studies in our own lab for RAS activation are currently ongoing and should provide some insight as to whether the observed effect of oestrogen on blood pressure may in part be explained by an effect on the RAS in this particular experimental model.

Oestrogen may also alter blood pressure by influencing sympathetic or parasympathetic activity. In the clinical setting it has been reported that pre-menopausal women have higher baroreflex sensitivity than age-matched men (Huikuri *et al*, 1996) and that this difference was attributed to the female sex hormone. Animal studies demonstrated that female rats that were chronically treated with oestrogen, at a dose equivalent to that during proestrous and additionally treated with an acute administration of oestrogen, had increased parasympathetic tone and an enhanced ability to resist increases in sympathetic output relative to male rats (Saleh and Connell, 1999). Although the dose of oestrogen used to supplement rats in this study was equivalent to that used in our study, the comparisons were drawn between distinct experimental groups and therefore it is difficult to apply the observations by Saleh *et al*. They compared male to female rats which were supplemented with oestrogen whilst we compared the effect of exogenous to endogenous oestrogen and also to oestrogen deprivation in female mice. It is not possible to exclude a role for oestrogen to increase blood pressure by exerting effects on the central nervous system in our model as the literature also reports that treatment of female rats with oestrogen decreased α_2- receptor expression in brain tissue by increasing G protein coupled receptor kinase (Ansonoff and Etgen, 2001). Therefore, it may be that relative to placebo-treated mice and sham ovx mice in our study, chronic oestrogen

treatment increased MABP by attenuating the reduction of sympathetic nervous system discharge that would otherwise be mediated via the α_2-receptor. Furthermore, the enzyme catechol-O-methyl transferase (COMT, reviewed in Mannisto and Kaakkola 1999) that metabolises endogenous (and exogenous) catecholamines, is also involved in the metabolism of estradiol (reviewed in Mannisto and Kaakkola, 1999). Therefore, in our mouse model, where exogenous oestrogen treatment increased blood pressure relative to endogenous oestrogen and placebo treated animals, competition between oestrogen and catecholamine for COMT may have resulted in an increase in the circulating levels of catecholamine. It is possible that the elevated levels of catecholamine result in a reduction in the sensitivity of central α_2- receptors and therefore attenuate the α_2- mediated reduction in sympathetic stimulation. This hypothesis is very speculative and deserves further investigation with more direct measurements.

We also found in this study that oestrogen deprivation lead to a reduction in MABP when compared to mice with either endogenous or exogenous oestrogen treatment. This finding may be surprising as a study by Javeshghani *et al*, reported that chronic oestrogen deprivation in mice, due to ablation of the gene for the follicle stimulating hormone receptor (FORKO mice) and therefore low levels of oestrogen, lead to an increase in MABP (2003). However, it is supported by the more recent study of Head *et al*, who demonstrated that female mice which were aromatase deficient, therefore unable to synthesis oestrogen, had a lower blood pressure than that of WT mice (2004). They reported a reduction in MABP of 4.5mmHg in ARKO mice relative to WT controls. Similarly, we found that MABP was reduced by 4.1mmHg in ovx placebo treated females when compared to female mice which were normally cycling. Although Head *et al* were unable to determine a mechanism for the reduction in MABP they suggested that it may be due to a role of oestrogen in maintaining normal vasoconstrictor activity (2004).

An additional feature of this study was the assessment of diurnal variation. We demonstrated that HR, blood pressure and activity increased at night, the natural active period, in each of the oestrogen treatment groups studied. Reports in the

literature show that fluctuations in levels of oestrogen during the oestrous cycle contribute to the preservation of the physiological circadian fluctuation of blood pressure. In a study by Mercuro *et al*, less than 10% of post-menopausal women studied had a peak- to- trough variation in blood pressure (1998). However, following oestrogen replacement therapy, the expected reduction in blood pressure at night was restored. Furthermore, in normally cycling female rats, the variation in the circadian system was shown to be greatest during proestrous at which stage in the oestrous cycle rats were also shown to be most active (Takezawa *et al*, 1994). Interestingly, we found in our study that although activity in each of the three groups studied increased at night, ovx female mice treated with either oestrogen or placebo were less active than normally cycling female mice. As the level of oestrogen used to supplement the oestrogen treated mice was equivalent to that during prooestrous it may have been expected that these mice would have been more active than either normally cycling or placebo treated animals. It is possible that in the oestrogen supplemented mice that the blunting of the increase of activity at night could be that the level of oestrogen is fixed and such diurnal variations are enhanced by fluctuations during the oestrous cycle. Alternatively, the reduced level of activity in oestrogen and placebo treated mice could be due to the additional surgery that these animals underwent, ovx and telemetry implantation as opposed to normally cycling female mice which underwent the telemetry implantation surgery only. However, this seems unlikely as the mice had recovered from ovx surgery, as determined by weight and daily inspection to ensure animals were healthy and infection free, before undergoing surgery for telemetry implantation.

In our study we also show that administration of the proprietary Organon ERß selective antagonist, increased MABP measured over 24hrs in sham ovx mice yet reduced MABP in ovx mice supplemented with oestrogen. This suggests therefore, that in normally cycling female mice, oestrogen acts through ERß to lower blood pressure and chronic administration of oestrogen acts through ERß to increase blood pressure.

Although this is the first study to show the effect of oestrogen in female mice on blood pressure and to demonstrate a role for ERß using the novel approach of a specific ERß antagonist, it is not the first time that a role for ERß in the regulation of blood pressure has been demonstrated. Zhu *et al* reported, using the ßERKO mouse as their experimental model, that loss of functional ERß lead to an increase in blood pressure, of ~10mmHg, in year old male and female mice, which were normally cycling (Zhu Y *et al*, 2002). In our study we observed a similar increase in MABP, 8.6mmHg and 6.2mmHg day and night respectively, in normally cycling female mice following treatment with the ERß antagonist. Furthermore, in rat, oestrogen acting centrally reduced the arterial pressure response to stress (Chernay *et al*, 2003). They demonstrated that these effects were in part mediated via NO which is known to reduce sympathetic output when applied to the paraventricular nucleus of the hypothalamus (PVN, reviewed in Kurkoff, 1999). This study was not designed to attribute this effect of oestrogen specifically through the action of one of its receptors. However, interestingly, immunoreactive ERß and mRNA have been described in the neurones of the PVN (Shughrue and Merchenthaler, 2001). Furthermore, ER gene expression has been demonstrated in neurones of the nucleus tractus solitaries (NTS) and caudal ventrolateral medulla (CVN) (Simerly *et al*, 1990) where the activity of neurones in these areas are also known to be affected by NO (reviewed in Kurkoff, 1999). The ERβ antagonist used in this study is predicted to be able to cross the blood brain barrier based on its physico- chemical properties, such as solubility and lipophilicity. Therefore from our study it may be suggested that the increase in blood pressure in mice which were normally cycling following treatment with the ERβ antagonist, may in part be due to the inhibition of ERß acitivity in the PVN. The inhibition of ERß in the PVN, NTS and or CVM may result in reduction in the release of NO.

The finding that the said ERβ antagonist reduced MABP in ovx mice administered oestrogen suggests that ERß is involved in the increase in blood pressure produced by chronic administration of oestrogen. This is in sharp contrast to our finding in sham ovx female mice and from that observed in the study by Zhu *et al* (2002) where ERß reportedly has an antihypertensive effect. To date the role of ERß in regulating

blood pressure has been restricted to mouse studies using either male or normally cycling female mice and studying the role of the vasculature in blood pressure. It is possible, that the higher concentration of oestrogen which results in an increase blood pressure relative to sham ovx mice also alters the functioning of ERß such that it acts to increase blood pressure. As previously discussed oestrogen may act to increase blood pressure by activating the RAS. The literature has not as yet identified a specific role for ERs in regulation of the RAS. However, Stavreus- Evers *et al*, reported that serum levels of angiotensinogen increased with increasing levels of oestrogen and ERα expression in the liver which was blocked by the non-selective ER antagonist ICI 182 780 (2001). To date expression of ERß has not been detected in the liver. Further investigation as to the mechanism by which chronic oestrogen treatment may increase blood pressure via ERß is required however it is possible that it may be due to a dose-dependent effect of oestrogen on the RAS.

In this study we also investigated vascular function as this may be one of the contributing factors through which oestrogen and ERß may influence blood pressure. The observed effect of oestrogen status and the ERβ antagonist on blood pressure in our study was mainly attributable to alterations in DBP which is indicative of alterations in vascular resistance. Oestrogen has been reported to lead to the alteration or modulation of ion fluxes, receptors on smooth muscle cells and modulation of endothelium-derived factor production and activity (White *et al,* 1995, Austin *et al* 1995). Therefore, we compared vascular responses in the mesenteric artery from mice of different oestrogen status as well as assessing vascular function in mice that had been treated with the said ERβ selective antagonist.

We showed that the contractile response to PE was similar between normally cycling female mice and those that had undergone ovx and treatment with placebo. However, female mice that had undergone ovx and were then supplemented with oestrogen had an enhanced contractile response to PE in comparison to those mice that were normally cycling. The increase in vascular tone, either as a result of enhanced vasoconstriction, impaired endothelium-dependent vasodilatation or a decreased

vascular sensitivity to NO, following chronic oestrogen administration may be one of the mechanisms by which blood pressure was increased in these animals.

The enhanced contractile response to PE following administration of exogenous oestrogen in this study was supported by the findings of Colucci *et al* and Cheng *et al* (Colucci *et al*, 1982 and Cheng *et al,* 1992) who reported that oestrogen treatment of female rats lead to an increase in the sensitivity to catecholamines in both isolated aorta and mesenteric arteries. It was suggested that the enhanced contractile response to catecholamines following oestrogen treatment may be explained by an increase in the affinity of vascular α-adrenergic receptors (Colucci *et al,* 1982), and by the inhibition of uptake, which is involved in the termination of catecholamine action, by estradiol (Salt, 1972, Hamlet *et al*, 1980, Gisclard *et al*, 1987). Alternatively, as shown by Bento and Moraes, oestrogen pre-treatment increased the number of α_1-adrenoceptors and the amount of intracellular calcium available for contraction (1992). Future experiments would include determining the effect of oestrogen on the contractile response to other contractile agents such as 5-HT or thromboxane analogues. This would provide some insight into whether the enhanced response following chronic oestrogen treatment was specifically due to enhancement of the α-adrenoceptor mediated response or due to sensitisation of contractile proteins. If the response was found to be specific to adrenoceptors, further experiments to elucidate the exact mechanism would be required. One such experiment could be to determine whether the increase in α- adrenoceptor sensitivity was due to the blocking of uptake. This could be tested by measuring the α- adrenergic response in the presence of cocaine, an uptake inhibitor. If the contractile response was not further enhanced by the presence of cocaine, or less so than vessels from sham ovx or those mice which were treated with placebo it would suggest that the increased sensitivity to the adrenoceptor agonist was due to oestrogen blocking uptake.

Another factor may be the concentration of oestrogen used to supplement animals and the stage of the oestrous cycle in which the sham ovx animals were at time of being euthanised. As discussed earlier, the plasma concentration of oestrogen achieved following supplementation (0.05mg/pellet) was $50pgml^{-1}$, which is closer to

the higher concentrations of oestrogen during the mouse oestrous cycle. In contrast the sham ovx animals included in this study were in stages of the oestrous cycle when concentrations of oestrogen were low. A study by Bolego *et al* reported that aorta isolated from female rats treated with low dose oestradiol ($5\mu gkg^{-1}$), which was equivalent to the levels of oestrogen during proestrous, had increased levels of basal NO whilst administration of high dose oestradiol ($100\mu gkg^{-1}$) inhibited the release of basal NO whilst also increasing the release of endothelium derived vasoconstrictor substances (1997).

It is difficult to draw comparisons between this study and ours as although the low dose they selected was physiological, it was equivalent to that used to supplement the animals in our study. The higher dose, at which alterations in vascular function occurred, was pharmacological. In contrast, we observed similar effects with different concentrations of oestrogen within physiological limits. We cannot determine from this study whether the difference in vascular response was due to low levels of oestrogen, as the animals in our study which were normally cycling were at stages of the oestrous cycle when oestrogen levels were low, or due to the fluctuations in oestrogen during the oestrous cycle. Further investigation to assess the contractile response to PE in vessels obtained from mice during different stages of their oestrous cycle would provide further insight.

Endothelial cells control vascular tone in response to physiological stimuli- sheer stress, pressure or pharmacological stimuli, through the synthesis and release of vasoactive agents that affect the contraction of the underlying vascular smooth muscle cells. To assess endothelial function and whether this could provide an explanation for the effect of oestrogen on blood pressure and indeed the vasoconstrictor response, we studied both endothelium -dependent and -independent vasodilation.

We found that the contractile response to PE was unaltered by the presence of L-NAME in normally cycling female mice and ovx mice administered either placebo or oestrogen. This suggests that in first order mesenteric arteries from female mice,

NO is not the main mediator of basal vascular tone. However, literature has reported that vasorelaxation of mouse mesenteric arteries results from activation of both the NO and COX pathways (Cooke *et* al, 2003a and Chataigneau *et al,* 1999). An explanation for the observation may be the difference in the sex of the animals studied as our studies in contrast to those were carried out in female mice. Interestingly, the involvement of these pathways has been suggested to be influenced by oestrogen status as endothelium- dependent relaxation of mesenteric arteries from pregnant mice, which have high levels of oestrogen, require inhibitors of both NO and COX pathways before the relaxation response is attenuated (Cooke *et al,* 2003b). This is in contrast to mesenteric arteries from non- pregnant mice, in which the endothelium- dependent vasodilatory response can be blocked by inhibitors of the NO pathway alone (Cooke *et al,* 2003b). Furthermore, the relative contributions of endothelium- derived vasodilators have been reported to vary with the size of the vascular bed studied. It was demonstrated in the cerebral vascular bed of the rat that oestrogen treatment increased endothelial NO production, in larger cerebral arteries, with no apparent effect on COX pathways whereas, in smaller cerebral arteries prostanoids play a more prominent role (You *et* al, 1999 and Ospina *et* al, 2003). Interestingly, we demonstrated that incubation with indomethacin slightly increased the contractile response to PE in mesenteric arteries from ovx mice treated with oestrogen. This suggests that chronic oestrogen treatment lead to an increase in the release of vasodilator prostanoids from the endothelium. However, this was not sufficient to counteract the mechanism by which oestrogen increases vascular sensitivity to PE. These findings suggest that as oestrogen acts to increase the release of vasodilator prostanoids from the endothelium and the response to PE was unaltered by the presence of L-NAME, the increased sensitivity to PE cannot be explained by a reduction in the levels of basal NO or alteration of prostanoid levels.

Although decreased levels of basal NO were shown not to be an explanation for the potentiated response to PE following oestrogen supplementation, the relaxation response to ACh and SNP were found to be reduced in vessels from these mice relative to those from sham ovx and placebo treated mice, which were not different to each other. As the response to SNP was reduced to a similar degree to that of

ACh, this suggests that the ability of vascular smooth muscle cells to relax in response to NO was impaired following ovariectomy and oestrogen supplementation. This may be due to down regulation or desensitisation of the components of the signalling pathway of NO in VSMC such as guanylate cyclase (GC) and cGMP following chronic oestrogen treatment. Such a hypothesis is supported by the report that incubation of cultured PC12 cells with oestrogen decreased cGMP levels (Chen *et al,* 2001). This was attributed to a reduction in soluble guanylate cyclase as the activation of cGMP in response to SNP was reduced whilst that to ANP was unaffected. Therefore, future studies on the vasculature of our mice such as western analysis to quantify the expression of soluble guanlyl cyclase, would be required to determine whether such a mechanism for oestrogen to reduce the sensitivity of vascular smooth muscle cells to NO could exist.

Although less likely, other possibilities for the reduction in the response to ACh could be due to alterations in the release of endothelium derived hyperpolarizing factor (EDHF). In this study we did not test the relaxant response to ACh in the presence of the NOS inhibitor L-NAME and therefore it is not possible to conclude that the relaxation response to ACh in the animals studied was solely due to alterations in the stimulated release of NO. It is also possible that the reduction in response to ACh was due to oestrogen reducing the release of, or smooth muscle sensitivity to, EDHF. Further experiments to determine whether oestrogen has modulated the release of NO, prostanoids or EDHF could be ascertained by determining the relaxant response to ACh in vessels in the presence of both indomethacin and L-NAME and whether such a relaxant response could be inhibited by charybdotoxin (ChTX), which would block the EDHF response. A role for oestrogen in regulating EDHF was demonstrated by Golding and Kepler, as they showed that EDHF mediated dilations in the cerebral artery were nonexistent in female rats which had undergone chronic oestrogen administration following ovx and endogenous oestrogen in intact female rats. This effect of oestrogen was confirmed by the observation that EDHF relaxation was enhanced in rats which had undergone ovx. Although there are some contradictory reports in the literature, demonstrating that oestrogen treatment enhanced relaxation induced by EDHF (Liu

et al, 2001), once again there are also indications that the cellular modes of action of EDHF show both tissue and species variability (Garland *et al,* 1995).

The observation in this study that treatment of sham ovx and ovx mice supplemented with oestrogen, with the ERß selective antagonist lead to an increase and decrease in blood pressure respectively, may in part be attributed to alterations in vascular reactivity of these animals.

In normally cycling female mice, the ERß selective antagonist did not alter the contractile response to PE or basal levels of NO or prostanoids in the mesenteric artery. However, the study by Zhu *et* al, which reported an increase in MABP in both male and female, normally cycling, ßERKO mice also reported that ERß attenuated vascular contraction as aorta isolated from these mice had an increased contractile response (2002). Therefore they suggested that the increase in MABP in the ßERKO mouse may at least in part be attributed to an increase in vascular tone. However, it is possible that the conflicting findings between the studies are due to differences in experimental approach between the selective ERß antagonist, in our study, to that of a knock out mouse model. In addition, although blood pressure studies by Zhu *et al*, were carried out in both male and female mice, the vascular study was done in aortic vessels from male mice. Therefore, it is difficult to compare results between our study and that of Zhu *et al* due to differences in both the sex of the mice and vascular bed studied. Although their paper reports an increase in the response to PE in denuded vessels, supplementary data revealed that this was only in aortic vessels in which the endothelium had been removed. Therefore similar to our findings in female endothelium intact first order mesenteric rings, they report that the contractile response to PE between WT and ßERKO mice in endothelium intact aortic rings from male mice was not different. Although, this was not further discussed in the paper this would suggest that the reactivity of the endothelium is altered such that it would mask differences in vascular smooth muscle function which would only become apparent when the endothelium was removed. Furthermore, endothelial function was not determined by the more typical method of L-NAME or indomethacin to determine the basal release of NO or prostanoids or determining the

endothelium- dependent and –independent vasorelaxation responses using ACh or SNP but rather to the vasodilatory response to acute administration of oestrogen.

In contrast to that observed in normally cycling female mice, treatment of female mice supplemented with oestrogen with the said ERß antagonist lead to a reduction in MABP, restoring BP to levels seen in sham ovx mice. We demonstrated that the sensitivity of VSMC of vessels obtained from these mice to PE was reduced when compared to vessels from mice which did not receive the antagonist suggesting that the reduction in blood pressure may be in part due to a reduction in vascular tone. To determine whether this effect of the ERß antagonist was due to alterations in the basal release of either NO or constrictor prostanoids, the response to PE was determined in the presence of L-NAME and indomethacin. We reported, similar to the observation in ovx mice supplemented with oestrogen which did not receive the antagonist, that incubation with L-NAME did not alter the contractile response to PE. Therefore, the increased sensitivity to PE in vessels in mice treated with the ERß antagonist remained in the presence of L-NAME and suggested that ERß does not modify the release of basal NO at least in the female mouse mesenteric vascular bed. However, we demonstrated that treatment of oestrogen supplemented mice with the ERß antagonist abolished the enhanced sensitivity to PE in the presence of indomethacin observed in vessels from those mice which did not receive the antagonist. This suggests therefore, that ERß may mediate the release of vasodilatory prostanoids by chronic administration of oestrogen. Therefore, the reduction in sensitivity to PE in vessels from oestrogen treated female mice following treatment with the ERß selective antagonist was not due to an increase in the basal release of NO or in the release of vasoldilatory prostanoids through ERß.

However, the relaxation response to ACh and SNP was increased following treatment with the ERß selective antagonist. We reported that chronic oestrogen supplementation reduced the vasodilatory response similarly to both ACh and SNP thereby suggesting a reduction in vascular smooth muscle sensitivity to NO which may be attributed to a reduction in soluble guanylate cyclase or activity of cGMP. Therefore it is possible that in mice supplemented with oestrogen, ERß attenuates

signalling of NO through the GC pathway, as following treatment with the antagonist the relaxant responses to ACh and SNP were restored. It is possible to suggest therefore that ERß in female mice supplemented with oestrogen, can modulate vascular tone by reducing sensitivity to NO.

In conclusion, the major finding of this chapter was that oestrogen supplementation of ovx mice increased blood pressure when compared to that of female mice which were normally cycling. Ovx mice treated with placebo had a reduced bp relative to normally cycling mice. The increase in MABP in oestrogen supplemented mice may be attributed in part to an increase in vascular sensitivity to PE and a decrease in endothelium-dependent and independent vasodilatation. Neither decreased NO, nor increased constrictor PG, bioavailability can account for these effects of oestrogen in the vessels. In addition, it was shown that all of the effects of oestrogen supplementation on bp and vascular function were reversed in mice treated concurrently with the ERß selective antagonist.

In contrast to the above, the ERß antagonist increased, rather than reduced BP, in normally cycling female mice, although there was no accompanying change in vascular function.

The opposite effects of the ERß selective antagonist on MABP in normally cycling female mice and those ovx and treated with oestrogen, suggests that different levels of oestrogen, or indeed, chronically sustained elevated levels of oestrogen, produce opposing effects whilst acting through the same receptor, perhaps due to modification of receptor expression under these different conditions.

Chapter 6

General discussion

As pre-menopausal women had a lower incidence of cardiovascular disease than either post-menopausal women or age- matched men, it was proposed that oestrogens protected against cardiovascular disease (Sullivan *et al,* 1990, Benetos *et al*, 1999). However, randomised controlled clinical studies have suggested not only that HRT provides no benefit but that in the first year of treatment there is an increased risk of cardiovascular events (Hulley *et al*, 1998, WHI 2002). A number of studies using a variety of animal models have reported that oestrogens, via actions on both the vasculature and heart, are cardioprotective.

When I started the studies described in this thesis, experiments of the effects of oestrogen on the cardiovascular system were largely focussed upon the use of mice with targeted disruption of the ERα and ERß genes. The use of these mice as animal models for oestrogen research had progressed despite a lack of knowledge about the cell specific pattern of expression of the two ERs in the mouse cardiovascular system. At that time research using ERKO and βERKO mice and their wild type control littermates had mainly focused on the effects of oestrogen on the vasculature, specifically vascular injury (Iafrati *et al*, 1997, Karas *et al*, 1999) and vascular tone (Rubanyi *et al*, 1997, Nilsson *et al*, 2000). These studies had demonstrated that chronic oestrogen supplementation may attenuate vascular smooth muscle cell proliferation via ERα or ERβ (Iafrati *et al*, 1997, Karas *et al*, 1999) and that endogenous oestrogen in male mice increased the basal release of NO via ERα (Rubanyi *et al*, 1997). However, it was only recently that characterisation of the role of ERβ in mediating the effects of oestrogen in the vasculature began. Nilsson *et al* (2001), in contrast to Rubanyi *et al* (1997), studied the relaxant response to acute administration of oestrogen and reported that oestrogen acting through ERβ negatively regulated the release of NO. Therefore, a distinct lack of clarity existed as to the individual effects of ERs in regulating vascular tone due, in part, to differences in the experimental design employed in the different studies. Furthermore, correlating with the onset of our study, the literature reported that a polymorphism in the human gene encoding ERβ was associated with hypertension in Japanese women (Ogawa *et al*, 2000). Therefore, the purpose of this study was to determine whether

oestrogens and in particular oestrogens acting through ERβ modify vascular function and contributes to the regulation of blood pressure.

In our initial investigations of the distribution and cellular localization of ERs in the cardiovascular system of WT mice we demonstrated that ERβ, but not ERα, could be immunolocalized to the nuclei of cardiomyocytes and nuclei of endothelial and vascular smooth muscle cells of vessels from both aortic and mesenteric vascular beds. In addition, cellular localisation and tissue distribution within the cardiovascular system was the same for both male and female mice. The pattern of distribution of ERβ was similar to that reported in the rat (Andersson *et al* 2001, Rodrigo *et al*, 2002). More recently, in support of our findings, Liang *et al* demonstrated that ERβ was expressed in mouse aorta by both immunohistochemistry (Liang *et al*, 2001) and Western blotting (Liang *et al*, 2003). Although in their original study Liang *et al,* in agreement with our observations, demonstrated that ERα was not expressed in mouse aorta (2001), they were able to detect it in protein extracts using Western blotting (2003) and therefore, this is something that we may wish to pursue in the future. Recently, Forster *et al* (2004), in contrast to our findings, reported that they were unable to detect ERβ in mouse hearts by either immunohistochemistry or Western blotting. The literature is rather conflicting as regards expression of ERβ in the heart with some studies reporting expression only in the nucleus of cardiomyocytes (Saunders *et al*, 1997) and others, restricted specifically to the mitochondria (Taylor *et al*, 2000). However, in support of our initial findings we were able to confirm that immunoreactive ERβ was expressed in the nuclei of cardiomyocytes using a second antibody which was directed against a different region of the ERβ protein.

Having determined that ERβ was expressed in the vasculature we aimed to investigate the physiological role of ERβ in mediating the effects of oestrogen in both male and female mice. In male mice, we studied the role of ERβ in mediating the effects of endogenous oestrogen in both young and aged animals using the ßERKO model. We demonstrated that loss of functional ERβ lead to an increase in vascular contractility to

PE in both young and aged animals. The enhanced vasoconstricting response to PE in young βERKO mice relative to age-matched WT controls could in part be attributed to impaired endothelial vasodilatation, as both the basal release of NO and the relaxation response to ACh was reduced in the vasculature of βERKO mice. This suggested that oestrogen acting through ERβ increased the bioavailability of NO. The studies described in this thesis were not designed to determine the exact mechanism by which this may occur. However, since our study several mechanisms have been suggested, from localisation of ERβ in caveolae leading to activation of eNOS (Chambliss *et al,* 2002), induction of iNOS expression (Zhu *et al,* 2002) and by activating anti-oxidant enzymes (Tamir *et al*, 2002, Montano *et al*, 2004). Whether such mechanisms applied to our model could be determined by measuring the levels of eNOS by Western blotting and by measuring tissue levels of NO. In vessels from aged animals, the loss of functional ERβ also resulted in enhanced sensitivity to PE. However, in contrast to young βERKO mice, there was no difference in the basal release of NO between aged WT and βERKO mice. This suggested that the enhanced vascular contractility in βERKO mice may also be due to a direct effect of ERβ on vascular smooth muscle cells, possibly via the regulation of ion flux. As the contractile response to KCl was similar between all the groups studied it was unlikely that the enhanced contractile response was due to alterations in Ca^{2+} influx. However, it has been demonstrated that oestrogen can regulate the expression of subunits of K^+ channels such that it enhances K^+ efflux (Benkusky *et al,* 2002). Therefore it is possible that, in βERKO mice, the loss of ERβ lead to impaired function of K^+ channels and subsequently K^+ efflux leading to enhanced vasoconstriction. Whether ERβ is the receptor responsible for the effect of oestrogen on K^+ channel subunits and if this could be a mechanism by which vasculature contractility is enhanced following the loss of ERβ could be investigated by measuring the expression of the K^+ channel subunit in both WT and βERKO mice. Preliminary studies using RT-PCR and toxinhistochemistry, carried out by a collaborating lab, demonstrated that loss of functional ERβ in our colony of βERKO mice lead to a trend developing for a reduction in the expression of the subunit of the K^+ channel in both brain and aorta. However due

to the low numbers of animals studied this did not reach significance. It would be interesting to be able to complete this study and determine whether the trend for a reduction in K^+ channel expression would reach significance. Although we demonstrated that loss of functional ERβ lead to enhanced vascular tone and impaired vasodilatation similar to the observations by Zhu *et al* (2002), in contrast, we did not find that impaired vascular function in the βERKO mouse lead to an increase in blood pressure. This may be due to the different techniques used to measure blood pressure between our studies. However, it was also possible that it was due to variations between the βERKO mouse model used in our studies. Whilst determining the expression of ERβ in the mouse cardiovascular system we found that we were able to detect immunoreactive ERβ in the βERKO mouse using antibodies directed against the hinge domain and the C-terminus of the protein. However, we also concluded that in these mice in which targeted disruption of an exon encoding the DNA binding domain has been undertaken the ERβ protein they expressed did not function in the same way as the WT protein as female βERKO mice presented with the same ovarian phenotype as described by Krege *et al* (1998). Western analyses of tissues from these mice are underway to elucidate what regions of the ERβ protein are expressed in the variant strain of βERKO mouse used in our study. However, it seems possible that this may provide an explanation for the differences observed between our study and that of Zhu *et al* (2002).

Due to the recent advances in the development of selective ER agonists and antagonists and the expression of residual ERβ protein in the βERKO mouse we decided to change our approach to studying the role of ERβ. Through collaboration with Organon we were able to use an ERβ selective antagonist to investigate the influence of oestrogen, and the role of ERβ, on vascular function and in the regulation of blood pressure, as assessed by radiotelemetry. In this study we found that in WT female mice, chronic oestrogen supplementation increased MABP when compared to both normally cycling and placebo treated female mice. As discussed in *Section 5.4*, this could be due to effects of

oestrogen on the RAS and this is currently being investigated in our lab by measuring plasma renin activity and angiotensin concentration. However, as the increase in MABP in this group of mice studied could be attributed to the concurrent increase in DBP it suggests that an increase in vascular tone could also be one of the mechanisms by which blood pressure was increased. Interestingly, the increase in blood pressure in the oestrogen treated group was reflected in the vascular study of first order mesenteric arteries where chronic oestrogen treatment resulted in a leftward shift of the PE CRC. Whilst investigating whether this was due to a reduction in basal NO, we revealed not only that basal NO was not the main vasodilator in the mesenteric artery of female mice but also that chronic oestrogen treatment lead to an increased release of vasodilating prostanoids from the endothelium. These findings were in contrast to those observed in the male study where NO was the main vasodilator and that prostanoids released from the endothelium lead to vasoconstriction. The explanation for this may not only be the sex difference in the animals studied but also the vascular bed as these have been reported to effect the release of vasoactive factors from the endothelium (Huang *et al*, 2001 and Sun *et al*, 1999, Ospina *et al*,2003). Results from the second arm of the vascular study in which aortic vessels from each of the groups studied has yet to be analysed. This should provide insight into whether the differences in the basal release of vasodilator between our two studies was indeed due to differences in gender or simply in the vascular bed studied. Despite oestrogen supplementation increasing the release of vasodilating prostanoids from the endothelium, it was not sufficient to attenuate the enhanced vascular tone. The response to both endothelium- dependent and –independent vasodilators was reduced in a similar manner in vessels from oestrogen supplemented mice relative to those from normally cycling and placebo treated animals. This suggested that chronic oestrogen supplementation reduced the ability of vascular smooth muscle cells to relax to NO. Therefore, in addition to the effect of oestrogen on the response to PE and that this is not attenuated by blocking either NO or prostanoids, it is possible that oestrogen treatment alters vascular smooth muscle cell function. Studies by Colucci (1982) and Bento and Moraes (1992) suggest oestrogen may increase the affinity or number of vascular α-adrenergic receptors. Ongoing experiments in our lab

will determine whether the enhanced vasoconstriction to PE is due to effects of oestrogen on adrenoceptors by studying the contractile response to different contractile agents. Preliminary data presents a trend for oestrogen treatment to shift the CRC to 5-HT to the left of normally cycling female mice. This is similar to that observed with PE and therefore suggests that enhanced vascular tone is not specific to adrenoceptors and subsequently that oestrogen may either increase the sensitivity of contractile proteins or as previously discussed alter ion flux which could be investigated by contracting vessels in the presence of a K^+ channel blocker.

In female mice which were normally cycling, treatment with the ERß antagonist lead to an increase in MABP. This was similar to the finding by Zhu *et al*, (2002) in which female ßERKO mice demonstrated a similar increase in MABP relative to WT females. Although the increase in MABP following treatment with the said antagonist was due to an increase in DBP we found that the increase in MABP was not reflected in vascular function of these mice. Therefore, results from ongoing experiments in the lab of levels of plasma renin activity in these animals may provide useful insight into the regulation of blood pressure in these animals. In sharp contrast, the treatment of mice supplemented with oestrogen with the ERß antagonist lead to a reduction in MABP. The vascular response to PE was also reduced in these animals despite the release of vasodilating prostanoids being attenuated. The vascular response to ACh and SNP was also improved suggesting that the ability of vascular smooth muscles to relax in response to NO is improved by blocking ERß. These findings suggest that the effects on the vasculature are independent of the endothelium and due to alterations in vascular smooth muscle cell function. Therefore, it will be interesting to test this hypothesis by determining whether the responses are similar in endothelium denuded vessels. Again it seems likely the reduction in vascular tone following treatment of mice supplemented with oestrogen and treated with the ERß antagonist, is not due to an affect on vascular adrenoceptors, as preliminary studies in our lab indicate that the contractile response to 5-HT in vessels from female mice supplemented with oestrogen are shifted to the right in vessels from animals that had also been treated with said antagonist. The opposite effects of the ERß

antagonist on MABP in normally cycling female mice and those ovx and treated with oestrogen, suggests that different levels of oestrogen, or indeed, chronically sustained elevated levels of oestrogen, produce opposing effects whilst acting through the same receptor, perhaps due to modification of receptor expression under these different conditions. Therefore, it will be interesting to determine whether expression of ERα and ERß is altered by differences in oestrogen concentrations. In addition, to further improve our understanding of the physiological significance of changes in ER expression by oestrogen, the actions of these receptors during different oestrogen status could be investigated through the use of ER selective agonists.

The hypothesis of this thesis was that oestrogen acting through ERβ modifies vascular function and contributes to the regulation of blood pressure. In summary, our findings are that ERβ, which is expressed in the myocardium and vasculature of both male and female mice, influences vascular function. In male mice, through the use of the ßERKO, we demonstrated that ERß mediates the effects of oestrogen as the loss of functional ERß lead to enhanced vascular contractility and impaired endothelial function. However, the increase in vascular tone and impaired endothelial vasodilatation were not implicated in the regulation of MABP. In female mice we found that oestrogen supplementation led to an increase in blood pressure when compared to that of female mice which were normally cycling, and those treated with placebo. This may be attributed in part to an increase in vascular sensitivity to PE and a decrease in endothelium-dependent and independent vasodilatation. ERß may influence blood pressure by altering vascular function as the effects of oestrogen on blood pressure and vascular function were reversed in oestrogen supplemented mice treated concurrently with the ERß selective receptor antagonist. By contrast, the said antagonist increased rather than reduced blood pressure in normally cycling female mice while vascular function was unaltered. This suggests that different levels of oestrogen, or indeed, chronically sustained elevated levels of oestrogen, produce opposing effects while acting through the same receptor.

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